MOLECULAR MECHANISMS THROUGH WHICH TICKS EVADE HOST DEFENSE

A Dissertation

by

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ABSTRACT

Ticks seriously affect mammals and immunization of host is considered as sustainable option for their management. Identification and validation of protective molecules are major challenges in developing vaccines against ticks. Based on understanding tick saliva-host interaction, efforts have been dedicated to tick control to diminish their deleterious effects. By utilization of tick saliva proteins previously identified by cDNA phage display library, transcriptomic and immune-proteomic studies, current study focuses on investigation of roles of 15 selected *Amblyomma americanum* and *Ixodes scapularis* recombinant tick saliva proteins (rTSPs) on host macrophage function. The effect of rTSPs on macrophage secretion of pro- and anti-inflammatory cytokines (TNF-α, IL-1, IL-6, IL-10, TGF-β) was investigated. Moreover, the functional role was examined by *in vivo* paw edema assay, cytokine and chemokine analysis. *In vitro* and *in vivo* investigations show that five *Amblyomma americanum* rTSPs, *Aam*IGFBP (Insulin like growth factor binding proteins) rP-1, *Aam*IGFBP-rP6 Short (S) and *Aam*IGFBP-rP6 Long (L), Serine protease inhibitor (serpin) 8 (AAS8) and *Aam*TCI (tick carboxypeptidase inhibitor), out of 15 are pro-inflammatory (PI) rTSPs as revealed by expression of pro-inflammatory costimulatory markers, cytokines, chemokines and signaling molecules. Interestingly, the two rTSPs serpins, AAS27 and AAS41, are anti-inflammatory (AI) rTSPs and appear to reverse the expression of co-stimulatory markers and cytokines induced by LPS and PI-rTSPs. Results indicate that

PI- and AI-rTSPs function in host evasion and thus may serve as potential candidate for anti-tick vaccination.

DEDICATION

This work is dedicated to my mother, Sania Feda Taj, and my father, Tajul Bakshi.

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It is my pleasure to acknowledge the roles of several individuals who were instrumental for completion of my PhD research.

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NOMENCLATURE

DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
CDC	Centers for Disease Control
h	Hour
IL	Interleukin
Th	T-helper cells
SERPIN	Serine Protease Inhibitor
TNF	Tumor necrosis factor
CD	Cluster of differentiation
TGF	Transforming growth factor

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CHAPTER I

INTRODUCTION

General Biology

Ticks are obligatory blood feeding ectoparasites that are distributed in nearly all habitable land (Sonenshine 1991). Ticks are classified in the order Ixodida, which contains three families: Argasidae, Ixodidae, and Nuttalleillidae. The family nuttalliellidae contains, which contains one species, *Nuttalliella namqua* is the least studied, and has only been reported in southern African countries of Tanzania, Namibia and South Africa (Keirans et al., 1976; Guglielmone et al, 2010; Mans et al., 2011). The other two tick families; argasidae and ixodidae are extensively studied, and have a global presence. The family argasidae, commonly known as "soft ticks" is subdivided into five genera containing at least 170 species. The family ixodidae commonly referred to as hard ticks is subdivided into five subfamilies that are organized into 12 genera and more than 650 species (Sonenshine 1991; Moon et al., 2002). Hard and soft ticks are so called, because of the texture of cuticle, which is hard and sclerotized for the former, and soft and leathery for the latter (Sonenshine 1991).

The tick life cycle has four stages: egg→larva→nymph→adult. Larva and nymph are commonly referred to as immature as they are not sexually developed. Except in the egg stage, each of the other tick life stages require a blood meal to develop to the next stage. For larvae and nymphs, they require a blood meal to molt to the next stage, while adult females take a blood meal to lay eggs and then die. Ticks infest a broad range of vertebrates, mammals, birds, reptiles and amphibians. Hard ticks are further

classified as one-, two-, or three- host ticks. One-host ticks spend their entire life cycle on the same host. For two-host ticks, both immature stages feed on the same host, while the adult tick feeds on a separate host. For three-host ticks, each tick feeding stage feeds on separate host. In addition to physical differences, hard and soft ticks are further distinguishable in how they feed. Whereas soft ticks take multiple blood meals for short periods of up to 5 min, hard ticks take a single blood meal that is completed over an extended period of 4-7 days for immature ticks (larvae and nymphs) and about 10-14 days for adult ticks (Sonenshine 1991).

Medical and Veterinary Importance

Medical and veterinary effects of ticks are described as direct or indirect, with the former mostly documented for hard ticks. Documented direct effects include paralysis and toxicosis, anemia, exsanguination in heavy tick infestations, stress, damaged skin impacting economic value of hides, and wounds at feeding site predisposing animals to secondary infections (Klompen et. al., 1996; Estrada-Peña and Jongejan, 1999; Jongejan and Uilenberg, 2004). Recent reports have documented devastating direct effects of tick infestations on wildlife (Fyumagwa et al., 2007; Keesing et al., 2013; Dantas-Torres 2015).

Although direct effects can lead to high economic loss, ticks are primarily known for their indirect effects or vector functions. Ticks can transmit multiple disease-causing pathogens including bacteria, protozoa, and viruses (Brites-Neta et al., 2015). Ticks outpace any other known arthropod vectors in terms of the diversity of transmitted disease agents, and they are considered second to mosquitoes in terms of the public

health impact of tick borne disease (TBD) agents. Medical and veterinary impacts of ticks and TBDs are well documented. In the livestock industry nearly 80% of the world's cattle are affected by TBD agents. Collectively losses due to direct effects of ticks and TBD infections amount to millions of US dollars annually (Jongejan and Uilenberg, 2004). Table 1 summarizes ticks and tick borne diseases of veterinary importance.

Table 1 List of tick borne diseases of veterinary importance

Tick vector	**Pathogen**	**Disease**	**Livestock/Domestic Animal**	**Endemic Countries**
Rhiphicephalus microplus; Ixodes ricinus; Haemaphysalis spp.*; Dermacentor* spp.*; Hyalomma* spp.*; R. sanguineus*	*Babesia bigemina; B. bovis; B. divergens; B. motasi; B. equi; B. caballi; Babesia gibsoni*	Babesiosis/Tick fever, Equine piroplasmosis	Cattle, Goats, Sheeps, Horses, Dogs	Central and South America, Europe, Africa, Australia and Asia
Argas persicus.; Ornithodoros spp.*; Dermacentor* spp..*; Ixodes* spp.*; Hyalomma* spp.*; Rhiphicephalus* spp.	*Anaplasma* spp.	Bovine, Equine, Canine Anaplasmosis	Cattle, Horses, Dogs	India, US, Africa, South America
Amblyomma spp. *R. sanguineus*	*Ehrlichia ruminantium*	Cowdriosis or Heartwater	Cattle, Goats, Sheeps	Southern, eastern and western African countries and in Madagascar
	E. canis; E. chaffeensis; E. ewingii	Ehrlichiosis	Dogs	India, US, Africa, South America, North America

Table 1 Continued

Tick vector	Pathogen	Disease	Livestock/Domestic Animal	Endemic countries
Ornithodorus moubata	African swine fever virus	African swine fever virus disease	Swine	Africa, Europe, Carribean
R. appendiculatus; H. anatolicum; Haemaphysalis spp.*; A. variegatum*	*Theilaria spp.*	East coast fever Tropical Theileriosis	Cattle, Sheep, goat	Eastern and southern Africa, Northern Africa, southern Europe, the Near East and the Far East, India
D. andersoni; D. variabilis; H. truncatum; I. holocylus; Rhiphicephalus spp.		*Paralysis Sweating sickness, Australian tick paralysis, tick toxicosis*	Cattle, Dogs, Cats	Australia, USA, Africa, South America, India
R. sanguineus	*Bartonella*	Bartonellosis, Cat scratch disease	Cats, Dogs	Europe, North America, Africa and China
Ixodes ricinus	Louping ill virus	Louping ill	Sheep/Grouse	British Isles
R. appendiculatus	Nairobi sheep disease virus	Nairobi sheep disease	Sheep	Africa
Hyalomma spp.	Crimean-Congo hemorrhagic fever virus	Crimean-Congo hemorrhagic fever	Cattle	Africa, Middle east and Asia
Dermacentor spp.; A. americanum	*Cytauxzoon felis*	Cytauxzoonosis	Cat	North America

Some of the most economically important TBD agents of livestock include the globally distributed *Rhipicephalus* (formerly *Boophilus*) *microplus* and *R. annulatus* transmitted bovine babesia parasites: *Babesia bovis* and *B. bigemina*, *R. appendiculatus* vectored *Theileria parva parva* in east and southern Africa, *Amblyomma variegatum*

transmitted *Ehrlichia ruminantium* in Africa, and *Anaplasma marginale* transmitted by multiple tick species including *Dermancentor* spp and *R. microplus* worldwide (Jongejan and Uilenberg, 2004; Marcelino et. al., 2012). Other important TBDs include equine piroplasmosis caused by *B. caballi* and *T. equi* that are vectored by *Dermacentor* spp ticks as well as *A. mixtum* (formerly *A. cajennense*) (Holbrook et al., 1968, Kerber et al., 2009). In companion animal health, important TBDs such as canine ehrlichiosis (Ewing et al., 1997), anaplasmosis, babesiosis (Kjemtrup and Conrad 2006), bartonnellosis (Breitschwerdt et al., 2010), and hepatozoonosis (Ewing and Panciera 2003), Lyme disease (Littman et al., 2006), Rocky Mountain Spotted Fever (Greene et al., 1985), Cytauxzoonosis (Reichard et al., 2009) have been documented.

Since the description of *Borrelia burgdorferi* as the causative agent for human Lyme disease in the 1980's there was a renewed focus on the impact of ticks in public health (Burgdorfer et. al., 1982; Johnson et. al., 1984). For instance, 15 previously unknown human TBD agents were reported between 1982 and 2001 (Parola and Raoult, 2001). At the time of drafting this dissertation, the US Centers for Disease Control (CDC) listed 16 reportable human TBD agents (https://www.cdc.gov/ticks/diseases) (Table 2). Moreover, the CDC, listed four other important human TBD agents including, the Crimean-Congo hemorrhagic fever, Kyasanur forest disease, Omsk Hemorrhagic fever and tick borne encaphalitis (https://www.cdc.gov/ticks/diseases/abroad.html). It is also interesting to note that, of the 24 vector borne disease agents that are listed by the United Nation's World Health Organization, seven (Crimean-Congo Hemorrhagic fever, Lyme disease, relapsing fever

borreliosisis, spotted fever and Q fever rickettsial disease, Tick-borne encephalitis, and tularemia) are tick borne (http://www.who.int/mediacentre/factsheets/fs387/en/).

Table 2 Tick borne diseases of the United States documented in humans (CDC, USA)

Tick species	**Causative agent**	**Disease**
Ixodes scapularis *Ixodes ricinus*	*Anaplasma marginale*	Anaplasmosis
Ixodes scapularis	*Babesia microti*	Babesiosis
Ixodes scapularis	*Borrelia mayonni*	Lyme disease
Ixodes scapularis	*Borrelia miyamotoi*	Lyme disease
Unknown	*Bourbon virus*	Bourbon disease
Dermacentor andersoni	*Coltivirus*	Colorado tick fever
Amblyomma americanum	*Ehrlichia spp.*	Ehrlichiosis
Amblyomma americanum	*Heartland virus*	Heartland virus disease
Ixodes scapularis *Ixodes pacificus*	*Borrelia burgdorferi*	Lyme disease
Ixodes scapularis *Ixodes cookei*	*Powassan virus*	Powassan disease
Amblyomma maculatum	*Rickettsia parkeri*	Rickettsiosis
Dermancentor variabilis *Dermacentor andersoni* *Rhiphicephalus sanguinineus*	*Rickettsia rickettsii*	Rocky mountain spotted fever
Amblyomma americanum	***Unknown***	STARI (Southern tick-associated rash illness)
Ornithodorus spp.	*Borellia hermsii* *Borellia parkeri*	Tick borne relapsing fever
Dermacentor occindentalis	*Rickettsia phillipi*	364D rickettsiosis
Dermacentor variabilis *Dermacentor andersoni* *Amblyomma americanum*	*Francisella tularensis*	Tularemia

Prevention of tick borne diseases

There had been attempts to develop effective vaccines against major TBD agents particularly for use in animals including vaccines against cattle *T. parva parva* (Radley et al., 1975), bovine babesiosis and anaplasmosis (Montenegro et al., 1992; Harper et al., 1994, Callow et al., 1997; Vercruysse et al., 2004). In public health, a vaccine against

the Lyme disease agent (Steere et al., 1998), which was commercialized over a brief period was withdrawn as model animal protective levels were not collaborated in vaccinated individuals (Rose et al., 2001; Lathrop et al., 2002). Thus, in the absence of effective vaccines against major TBD agents, prevention of animal and human TBD has depended on killing ticks. Although other approaches such as environmental management (Pavela et al., 2016) and biological methods (Kirkland et al., 2004a; Kirkland et al., 2004b; Hartelt et al., 2008) of ticks has been attempted to control tick infestations, killing ticks using acaricides has been the most reliable (Awumbila 1996; Holdsworth 2005). Despite the usage of these acaricides being effective in short term, they do not offer a permanent solution due to variety of serious limitations that include ticks developing resistance against multiple chemical acairicides and environmental contamination (Abbas et al., 2014; Brito et al., 2011; Guerrero et al., 2012; Rosario-Cruz et al., 2009). Tick development of resistance against tick infestation is particularly serious as costs for development of novel acaricide chemistry were estimated at over $100 million (Graf et al., 2004; de la Fuente et al., 2007). If acaricide based tick control methods were to fail, there would be catastrophic losses in the livestock industry and an unprecedented number of human TBD cases. Due to serious limitations of acaricide based tick control, alternative tick control methods have been documented (Mulenga et al., 1999; 2001; Willadsen et al., 1988; 1995; 2004; de la Fuente and Merino 2013). Immunization of animals against tick feeding has been validated as an effective and sustainable alternative tick control method (Rodríguez et. al. 1995; Willadsen et. al., 1995; Kumar et. al., 2009; Canales et. al., 2009; Merino et. al., 2011; Mulenga et. al.,

1999; Galaï et. al., 2012; Saimo et. al., 2011; Sugino et. al., 2003; Prevot et. al., 2007).

Immunization of animals against tick feeding: The need for effective anti-tick vaccine antigens

The concept of immunizing animals against tick feeding was based on observations that repeated infestation of rabbits by *Dermacentor variabilis* larvae provoked strong immunity that significantly reduced feeding performance of subsequent tick infestations (Trager et al., 1939). This work suggested that molecules existed in the tick that could be targeted for anti-tick immunization. These observations were reproduced by several other investigators (Kumar et al., 2009, Rechav and Dauth 1987, Schwartz et al., 1990, Shapiro et al., 1986) further confirming that tick molecules could be targeted for anti-tick vaccine development. Work by Australian scientists leading to the first commercialization of an anti-tick vaccine against *R. microplus* (Willadsen et al., 1995), TickGARD® Plus, provided credence to the concept of anti-tick immunization. Furthermore, scientists in Cuba validated the effectiveness of anti-tick immunization when they commercialized Gavac Plus®, a TickGARD sister vaccine as both vaccines were essentially based on the same antigen target molecule, Bm86 (De la Fuente et al., 1999, Valle et al., 2004). The limitations of both TICKGARD plus and GAVAC plus were quickly realized (De la Fuente, 2007): being effective against a single species and apparently tick strain specific as the vaccine appeared was not effective across geographic regions (De la Fuente, 2000). Additionally, immunizations with Bm86 homologs in other tick species did not confer protective anti-tick immunity (De Vos et al., 2001). Thus, the search for effective target anti-tick vaccine antigens in other tick

species was justified. The availability of effective anti-tick vaccine antigens is recognized as the limiting factor toward development and adoption of anti-tick immunization as the global strategy for anti-tick immunization (De la Fuente, 2007; Mulenga et al., 2001; Radulovic et al., 2015; Lewis et al., 2015; Kim et al., 2016; Tirloni et al., 2014; 2016). Without successful feeding, ticks can neither cause damage to its host nor transmit TBD agents. Thus, there has been a renewed focus to understand the molecular basis of tick feeding physiology as a means through which effective target anti-tick vaccine antigens can be discovered (Oliveira et al., 2013; Tirloni et al., 2014; Shwarz et al., 2014; Villar et al., 2014; Lewis et al., 2015; Kim et al., 2016).

Understanding the molecular basis of tick feeding physiology as a means of identifying effective anti-tick vaccine antigens.

Ticks accomplish blood meal feeding via pool feeding. Ticks feed by disrupting host tissue and sucking blood that collects into the wounded area, also referred to as the "tick-feeding lesion" (Sonenshine, 1991). This feeding style triggers host tissue repair and immune reactions that include inflammation, blood clotting, and complement activation systems that are aimed at stopping further blood loss and initiating tissue repair. Ticks overcomes host defenses, successfully feed, and transmit TBD agents by secreting diverse pharmacologically active agents in their saliva to suppress host defenses (Ribeiro 1989). Several lines of evidence have shown that tick salivary secretions include molecules that promote tick feeding and transmission of TBD agents. There have been considerable research interests in discovery of tick molecules that regulate tick feeding as these represent potential targets for anti-tick vaccine

development. Table 3 provides a summary of studies in ticks and host interactions over 10 years prior to start of my dissertation in 2013, while Table 4 summarizes studies during the five years of my PhD dissertation research. Collectively these studies demonstrate that the majority of our knowledge on tick and host interactions utilized crude tick protein extracts with a limited number of studies utilizing recombinant tick proteins. Toward identification of proteins that ticks inject into the host during feeding, there has been a concerted effort to determine molecular identities of tick proteins. Some of the described tick feeding promoting activities in tick saliva have included vasodilators that are thought to increase blood flow to the feeding site (Ribeiro et al., 1992), anti-haemostatics to prevent blood clotting (Ribeiro et al., 1985; Mulenga et al.,2013; Ibelli et al., 2014; Kim et al., 2015), anti-complement factors (Franco et al., 2016, Hourcade et al., 2016, Barratt et al., 2011), immune-suppressants (Juncadella et al., 2007), and inhibitors/modulators of innate and adaptive immunity (Cavassani et al., 2005, Vachiery et al., 2015; Scholl et al., 2016). This has been aided by recent publication of the *I. scapularis* (Hill and Wikel, 2005; Gulia-Nuss et al., 2016) and *R. microplus* (Geurrero et al., 2006) genomes. In other studies, tick salivary gland transcriptomes (Aljamali et al., 2009; Karim et al., 2011; Ribeiro et al., 2017; Moreira et al., 2017), immuno-proteomes/transcriptomes (Lewis et al., 2015), and tick saliva proteomes (Oliveira et al., 2013; Tirloni et al., 2014; 2015; Shwarz et al., 2014, Villar et al., 2014, Kim et al., 2016; Mudenda et al., 2014) were published. These studies have contributed to our understanding on molecular identification of proteins that the tick may inject into the host to regulate tick feeding and facilitate transmission of TBD agents.

The next phase of tick feeding physiology research and the focus of this dissertation is in part to understand the functional roles of proteins that the tick injects into the host in tick feeding regulation. This knowledge will likely reveal proteins that can be targeted for development of novel methods to prevent transmission of TBD agents. In this dissertation research, cell culture and *in vivo* approaches were utilized to define the role(s) of 15 recombinant tick saliva proteins in tick feeding regulation as revealed by effects on macrophage function. This dissertation has contributed to our understanding of how ticks evade the host's defense mechanisms.

Table 3 List of studies based on immuno-modulatory effects of tick saliva (Year 2003-13)

Tick species	Tick molecule	Host reaction	Host molecule	References
Rhiphicephalus sanguineus	Saliva (engorged)	Resistant animal: Induce basophils and neutrophils infiltration Susceptible animal: Effect delayed type hypersensitivity (DTH), basophils and mononuclear cell infiltration, protective immunity	Basophil and neutrophil infiltration/ DTH	Ferreira et al., 2003
Hyalomma anatolicum	Feeding (F)	Affect T cell proliferation	Increased CD4+/CD8+ T cells ratio, increase in circulating B cell count	Boppana et al., 2004
Ixodes ricinus	Saliva (Partially fed)	Inhibit B cell proliferation (LPS/ Borrelia OSP C induced)	B cells	Hannier et al., 2004
Rhiphicephalus sanguineus	Saliva (engorged)	Inhibition of bone marrow derived differentiation, maturation and function induced by LPS	Suppress cell surface marker expression of B cell: CD40, CD80 and CD86, CD54	Cavassani et al., 2005
I. ricinus	Salivary gland (SG) (partially engorged)	Suppress Cytokine production in susceptible mice	Epidermal cells: Downregulate TNFα and increase Th2 cytokine IL4 in C3H mice	Pechova et al., 2004
Dermacentor reticulatus *Amblyomma variegatum* *I. ricinus*	SG	Anti-cytokine activity	IL-8, MCP-1, MIP-1α, RANTES, eotaxin, IL-2 and IL-4	Hajnicka et al., 2005
D. andersoni	Saliva (4-5 days infestation)	Induce extramedullary erythropoiesis	Spleen: High CD49, low CD11a	Dash et al., 2005
D. andersoni *I. scapularis*	SG (Partially fed)	Reduce adhesion molecule expression ICAM and VCAM	Endothelial cells : *D. andersoni* : downregulate ICAM-1; *I. scapularis*: Downregulate P-selectin and VCAM-1	Maxwell et al., 2005
I. ricinus	Saliva, SG (Partially fed)	Anti-inflammatory activity	TNFα inhibitory activity	Konik et al., 2006

Table 3 Continued

Tick species	Tick molecule	Host reaction	Host molecule	References
I. ricinus	Saliva (Partially engorged)	Th2 immune response	CD4+ T cells: Increase in IL-4 cytokine leads Th2 immunity	Mejri and Brossard 2007
A. variegatum	SG, Females: 2&8 days, Males: 3, 7 and 11 days	Altered chemokine and cytokine activity	Anti- chemokine CXCL8, MCP-1,MIP-1, CCL3, CCL11	Vancova et al., 2007
R. sanguineus	SG: Evasin 1	Neutralize chemokine activity	CCL3, CCL4	Fraenschuh et al., 2007
I. scapularis	SG, Prostaglandin E2	Effect dendritic cell (DC) function	CD40, CD86, MHC II, CD4+T cell proliferation, IL-2	Sa-Nunes et al., 2007
R. sanguineus	Saliva (fully engorged)	Dendritic cell migration	Macrophage inflammatory protein α (MIPβ), T cell, CCR5	Oliviera et al., 2008
I. ricinus	Salp15	*Borrelia burgdorferi* induced Pro-inflammatory activity of Dendritic cells	Effect molecule: DC-SIGN, decrease IL-6, TNFα on Dendritic cells	Hovious et al., 2008
I. ricinus	SG (Partially engorged)	DC migration, maturation and function	Suppress B7-2 molecules, Induce Th2 response cytokines: IL-2, IL-4, IL-17	Skallova et al., 2008
R. sanguineus	Evasin 1, 3, 4	Chemokine inhibitory properties	Evasin 1: Suppress CCL3, CCL4, and CCL18; Evasin 3: suppress CXCL8 and CXCL1	Deruaz et al., 2008
A. cajannense	Saliva	Inhibition of Lymphocyte proliferation	Induce Th2 response	Castagnolli et al., 2008
I. scapularis	Saliva	Th1 inhibiton	Langerhan cells	Vesely et al., 2009
I. scapularis	Sphingomyelinase like enzyme	Th2 response	Cytokine Il-4 expression	Alarcon-Chaidez et al., 2009
R. sanguineus	Saliva	Inhibits maturation of DC	Inhibit ERK and MAP kinase signaling molecules, induce IL-10	Oliveira et al., 2010
R. microplus	Saliva, dopamine induced; F	Adhesion of PBMCs and endothelial cell migration	Resistant animal: high E selectin, Susceptible animal: high ICAM, VCAM, P selectin	Carvalho et al., 2010
I. ricinus	Saliva	Inhibition of proliferation and cytokine production	Decrease in virus induced TNFα and IL-6	Fialova et al., 2010

Table 3 Continued

Tick species	Tick molecule	Host reaction	Host molecule	References
A. variegatum, R. appendiculatus, D. reticulatus	SG (various days of feeding)	Affect Neutrophil chemoattractant property	CXCL8, CXCL1	Vancova et al., 2010
R. microplus	SG	Th1 > Th2 polarization	Macrophages: Increase in CD86 expression	Brake et al., 2010
D. variabilis	Saliva (Partially engorged)	Suppresses phagocytosis of macrophages; Th2 response	Macrophages : Induce IL-4 and IL-10 cytokine response	Kramer et al., 2011
R. sanguineus	Saliva (Engorged)	DC differentiation, cytokine production and costimulatory molecule expression	Inhibition IL-12p40, TNFα, increase IL-10	Oliviera et al., 2011
I. ricinus	Saliva (Partially engorged)	Decrease number of phagocytosing DCs	Inhibition of TNFα, IL-6 and increase in IL-10, Borrelia exposed DC suppress proliferation of DC and Il-2 production.	Slamova et al., 2011
I. ricinus	Salp15	Inhibit Keratinocyte inflammation	Chemokine IL-8, MIP and antimicrobial peptide downregulation	Marchal et al., 2011
I. ricinus	*Ixodes ricinus* 2 (IRS-2)	Inhibit paw edema formation	Cathepsin G and Chymase	Chmelar et al., 2011
I. ricinus	Saliva	IFN signalling in DC	STAT-1, Interferon β pathway	Lieskovska and Kopecky 2012
I. scapularis	Sialostatin L, cysteine protease inhibitor	Inhibits Th9 derived IL-9 production	IL-9 inhibition	Horka et al., 2012
R. microplus	SG, (Partially fed)	Macrophage responses	Inhibition of LPS induced macrophage response: Suppress CD80, CD86, CD40, IL-12, TNFα expression	Brake and Perez 2012
I. ricinus	Saliva (Partially engorged)	DC response signalling pathways	Suppression of Erk1/2, NFKB and Akt signalling molecules	Lieskovska and Kopecky 2012
I. scapularis	Saliva (Partially fed)	Suppress inflammatory response by macrophages	Decreasing levels of IL8, TNFα, IL12, IL6	Chen et al., 2012
I. scapularis	Saliva: 3, 6 and 12 h	Neutrophil driven response at bite site	Inflammatory genes	Heinze et al., 2012

Table 4 List of studies based on immuno-modulatory effects of tick saliva (Year 2013-17)

Tick species	**Tick molecule**	**Host reaction**	**Host molecule**	**References**
A. americanum	Serine protease inhibitor 6 (AamS6)	Anti-haemostatic functions	Serine and papain like cysteine proteases	Mulenga et al., 2013
R.appendiculatus	Japanin	DC response	Alter co-stimulatory molecules, co-inhibitory transmembrane molecules, pro-inflammatory/ anti inflammatory cytokine	Preston et al., 2013
D. variabilis	Saliva (partially fed), Prostaglandin E(2)	Macrophage migration and cytokine expression	Decrease pro-inflammatory cytokine; recruit fibroblast	Poole et al., 2013
I. scapularis	Serine protease inhibitor IxscS-1E1	Anti-haemostatic and anti-inflammatory	Cathepsin G, thrombin and trypsin	Ibelli et al., 2014
A. americanum	Calreticulin	No affect on plasma clotting and platelet aggregation	Bind complement molecule C1q	Kim et al., 2015a
A. americanum	Serine protease inhibitor 19	Anti-coagulant activity	Inhibit trypsin, plasmin, factor Xa and factor XIa	Kim et al., 2015b
I. scapularis	Sialostatin L2	IFN response in dendritic cells	IFNβ stimulated genes: Interferon regulatory factor 7 and IP-10 suppression	Lieskovska et al., 2015
I. ricinus	Saliva, (partially engorged)	Effect production of chemokine and cytokine response in mouse spenocytes	Upregulate monocyte chemoattractant protein (MCP-1), thymus derived chemotactic agent 3 (TCA-3) and macrophage inflammatory protein (MIP-2)	Langhansova et al., 2015)
A. americanum	Saliva, (Engorged tick)	Dendritic cell differentiation	Inhibit CD40, CD80, CD86, MHCII, CCR5, CCR7,IL-12, IL-6 and TNFα, increase PD-L1	Carvalho-Costa et al., 2015
I. scapularis	Sialostatin L and SialostatinL2	Dendritic cell response to *Borrelia* spirochetes	MIP-1α suppression, signalling molecules NfKb P13/Akr pathway suppression by SialoL2	Lieskovska et al., 2015

Table 4 Continued

Tick species	Tick molecule	Host reaction	Host molecule	References
A. maculatum	Infected Saliva with *Rickettsia parkeri*	Inflammation, T cell response	Lower inflammatory leukocytes, elevated C reactive protein, Th1 and pro-inflammatory cytokine in tick only animal	Banajee et al., 2015
A. variegatum	Saliva (partially fed)	Impaired monosyte recruitment, poor mobilization of monocyte derived cells from skin to lymph nodes	Dendritic cells: CD11c	Vachiery et al., 2015
I. scapularis	Saliva	Monocytic transcript level, pro-inflammatory mediators	Increased IL10, decrease in IL-8, IL-6 and TNFα, TLR2 expression	Scholl et al., 2016
A. maculatum	Saliva	Downregulation of acute cellular and cytokine immune response	Cutaneous cellular infiltration inhibition during acute Ricketsial infection	Banajee et al., 2016
A. americanum	AAS19	Binding with Heparin/heparin sulfate increase inhibitory activity of serine proteases of host; anticoagulant activity	Thrombin, factor Xa and factor XIIa	Radulovic et al., 2017
I. ricinus	Saliva	Effect chemokine, cytokine and leucocyte markers in skin	Early tick bite skin: CCL2, CCL3, CCL4 predominance and CXCL1 and CXCL8, IL-1 and IL-5. Lymphocytes and lymphocyte chemoattractant pro-inflammatory cytokines did not differ Later tick bite skin: immune cells and mediator declined and lymphocytes increased.	Glatz et al., 2017
I. ricinus	SG	Primary mast cells	Mast cell activation inhibition	Bernard et al., 2017
Ornithodorus moubata	Cystatin	Dendritic cell maturation	Target cathepsin S and C associated with maturation of dendritic cells	Zavasnik et al., 2017

Research significance

As summarized in Table 1, *A. americanum* and *I. scapularis* transmit a combined 11 of the 16 human TBD agents that are reported to the CDC. In our lab, *A. americanum* and *I. scapularis* transcriptomes (Radulovic et al., *in preparation*), immuno-transcriptomes (Radulovic et al., 2014; Lewis et al., 2015), *I. scapularis* and *A. americanum* saliva proteomes (Kim et al., 2016 and Kim et al., *in preparation*) were determined. In this PhD dissertation research, I have advanced our understanding of functional roles of some tick saliva proteins that these two medically important tick species inject into the host to regulate tick feeding. Results in this dissertation will lead to better design of anti-tick vaccine antigens. In addition to functional analysis associated with tick feeding events, these salivary proteins may be important in pathogen transmission. Thus, the overall success of this research will have broader impacts on tick vaccine development against tick feeding and pathogen transmission.

CHAPTER II

CHARACTERIZING EFFECTS OF *AMBLYOMMA AMERICANUM* AND *IXODES SCAPULARIS* RECOMBINANT (R) TICK SALIVA PROTEINS (TSP) ON MACROPHAGE FUNCTION

Introduction

Hard ticks are the major vectors of many pathogens, outpacing any known arthropod vector. In the livestock industry, losses due to ticks and tick-borne diseases (TBD) are estimated in millions of US dollars (Jongejan and Uilenberg., 2004). In North America, many human vector-borne diseases are primarily tick borne (Nathavitharana et al., 2015). The USA Centers for Disease Control (CDC) lists 16 reportable human TBD causative agents (http://www.cdc.gov/ticks/diseases/), 13 of which are transmitted by hard ticks. Globally the impact of TBD in public health has been on the rise, with the food-for-thought article on One Health listing human TBDs among sources of human health concerns needing One Health solutions (Dantas-Torres et al., 2012). Seven of the 24 vector borne disease agents that are listed by the World Health Organization ((http://www.who.int/mediacentre/factsheets/fs387/en/) are tick borne. These include Crimean-Congo Hemorrhagic fever, Lyme disease, relapsing fever borreliosisis, spotted fever and Q fever rickettsial disease, Tick-borne encephalitis, and tularemia.

Since there are no effective vaccines against major TBD agents, tick control using acaricides remains the major method to protect animals and humans against TBD agents (Awumbila 1996; Holdsworth 2005). Serious limitations such as environmental contamination and ticks developing resistance that threaten acaricide based tick control

have justified the need for alternative tick control methods (Graf et al., 2004; Abbas et al., 2014; Coles and Dryden, 2014). Immunization of animals against tick feeding has been validated as an alternative tick control method to solve the problems associated with acaricide based tick control strategies (Marcelino et al., 2012). The attraction to this approach is that it is environmental friendly and it is postulated to be effective against both susceptible and acaricide resistant tick populations. The limiting step is the availability of effective target antigens. With the exception of a few instances when human TBD infections occurred after exposure to contaminated materials (Liu et al 2012; Annen et al., 2012; Atkinson et al., 2013; Townsend et al., 2014; Jiao et al., 2015; Richards 2015; Yadav et al., 2016), both animal and human TBD infections require a successful tick bite. From this perspective a deeper understanding of tick feeding physiology as a means of finding novel anti-tick targets offers promise to develop new tick control method has been documented (Mulenga et al., 2007; Kim et al., 2016, Radulovic et al., 2014; Lewis et al., 2015; Porter et al., 2015; 2016; Tirloni et al., 2014; 2015; Ribeiro et al., 2006, Ribeiro et al., 2017; Karim and Ribeiro 2015; Garcia et al., 2014; Karim et al., 2011; Francischetti et al., 2008; Konnai et al., 2011; Mudenda et al., 2014; Diaz-Martin et al., 2013, de la Fuente et al., 2015).

One of the major adaptations that make ticks successful is their immense capacity to suppress host defense mechanisms to tick feeding. Hard ticks accomplish feeding by tearing up host tissue and sucking blood that bleeds into the feeding lesion (Bowman et al., 1997; Wikel 1999). This feeding style provokes host defense reactions such as inflammation, complement activation, platelet aggregation, and blood clotting

that are aimed at stopping blood loss and initiating tissue repair (Ribeiro et al., 2003; Francischetti et al., 2002; Kazimirova 2007; Kazimirova et al., 2003; Mulenga et al., 2001). Ticks successfully feed by secreting numerous saliva proteins that suppress host defenses.

In our lab, we are interested in identification and characterization of tick saliva proteins (TSPs) that regulate early stages of the tick feeding process. To this end we have used several approaches including western blotting analysis (Mulenga and Khumthong 2010; Kim et al., 2015a; Ibelli e et al., 2014; Kim et al., 2015b; Kim et al., 2016) and bio-panning of *I. scapularis* and *A. americanum* phage display cDNA expression libraries using antibodies to TSPs to determine tick saliva immuno-proteomes (Lewis et al., 2015; Kim et al., 2014). Recently we have also used Liquid chromatography-mass spectrometry sequencing of tick saliva proteins to identify *I. scapularis* and *A. americanum* tick saliva proteins that are sequentially secreted every 24 h during blood meal feeding (Kim et al., 2016, *in preparation* for *A. americanum*). Prior to the start of this dissertation, several TSPs were expressed as recombinant (r) proteins in yeast or insect cells: published (Kim et al., 2014; Kim et al., 2015b, Radulovic et al., 2015) and unpublished (Table 7). The goal of the second chapter of this dissertation was to gain insight into functional roles of selected *Amblyomma americanum* (n=12) and *I. scapularis* (n=3) rTSPs in tick evasion of host defense as revealed by macrophage functions.

Macrophages (MΦ) play important roles in host defense against ticks and tick borne infectious agents. They are important in the innate defense response and are

second to neutrophils to reach the infection site (Kumar and Sharma 2010). MΦ are the major regulators of the inflammatory response to injury and infection, acting as a bridge between innate and adaptive immunity (Getz 2005). They are essential immune cells because they monitor the external agents through an array of cell surface receptors and secrete various cytokines and chemokines that direct functions of other immune cells (Sternberg 2006). There is evidence that MΦ are among different immune cell types that infiltrate the tick feeding site (Silva et al., 2004; Boppana et al., 2005; Grigor'eva 2001) suggesting their involvement in tick and host interactions. In cell culture assays, crude tick salivary gland extracts were demonstrated to have taxis effects on MΦ suggesting that either ticks pro-actively attract this cell type to the feeding site or that the MΦ is among principal cells to the host's defense response to tick feeding (Poole et al., 2013). On this basis, the effects of candidate rTSPs on MΦ function could conceivablyprovide insights into functional role(s) of candidate TSPs in tick feeding regulation.

Materials and Methods

Expression of recombinant (r) tick saliva proteins (TSP)

Prior to the start of this dissertation research, fifteen rTSPs were expressed using the pPICZα plasmid and *Pichia pastoris* yeast cells and the pIB/V5-His plasmid and *Spodoptera frugiperda, Sf*9 or *Sf*21 insect cell expression systems as described (Mulenga et al., 2013; Kim et al., 2014; Kim et al., 2015b, Radulovic et al., 2015). Yeast and insect cell-expressed rTSPs were expressed from existing yeast and insect cell stocks. For yeast systems, stocks were cultured at 28°C with protein expression induced by adding 5% methanol to cultures daily. The pPICZα plasmid and *P. pastoris* yeast expression system

secretes recombinant proteins into media. For purification, rTSPs were precipitated from spent media using ammonium sulfate precipitation as previously described (Mulenga et al., 2013; Kim et al., 2014; Kim et al., 2015b, Radulovic et al., 2015). Precipitated rTSPs were resuspended in appropriate buffer (1 X Phosphate Buffered Saline, 50mM Tris 150mM NaCl or 200mM Tris, 1M NaCl, 10mM imidazole, pH 7.4) and dialyzed against affinity column binding buffer (200mM Tris, 1M NaCl, 10mM imidazole, pH 7.4). Likewise, the pIB/V5-His plasmid and *Sf*9 or 21 cell expression system secretes recombinant proteins into spent media. In the insect cell system, rTSPs were routinely concentrated using the using Jumbosep centrifugal spin filter devices with a 10 kD molecular weight cut-off point (Pall Life Sciences, Port Wahington, NY) as ammonium sulfate precipitation was not working well. Following concentration, insect cell expressed proteins were dialyzed against affinity column binding buffer (200mM Tris, 1M NaCl, 10mM imidazole, pH 7.4).

For both yeast and insect cell expressed rTSPs, the crude proteins was diluted with binding buffer and affinity purification was routinely done under native conditions using a Hi-trap $NiCl_2^+$ chelating HP columns (GE Healthcare Bio- Sciences Corp, Pittsburgh, PA). To verify purification and background contamination, affinity purified proteins were resolved on a 10% SDS–PAGE gel and silver stained using standard protocols (detailed below). The affinity-purified proteins were then concentrated, dialyzed against 1X PBS, pH 7.4) and stored −80°C until used for assays. Protein quantification was done using the Bicinchoninic acid (BCA) protein assay according to instructions by the manufacturer (Thermo Scientific, Wilmington, DE). Prior to use on MΦ cells (below),

yeast and insect cell expressed affinity purified rTSPs were depleted of endotoxins using a Pierce Lal Endotoxin removal kit (Thermo Scientific, Wilmington, DE).

Expression of rTSP in mammalian cells

In the preliminary screen, five of the 15 candidate rTSPs stimulated MΦ (detailed below) to express pro-inflammation markers. To rule out the possibility of endotoxin involvement in the observed results, the five (r*Aam*IGFBP-rP1, r*Aam*IGFBP-rP6 long (L), r*Aam*IGFBP-rP6 short (S), rAAS8 and r*Aam*TCI) rTSPs that stimulated MΦ were re-expressed in Human Embryonic Kidney (HEK) mammalian cells using the pcDNA 3.3 expression plasmid (Thermo Scientific, Wilmington, DE). The pcDNA 3.3 was modified (kindly provided by Dr. Mwangi) to include the CD5 secretion signal to allow rTSPs to be secreted into spent media (Njongmeta et al., 2012). Mature TSP open reading frames (ORF) were sub-cloned into the modified pcDNA plasmid using primers listed in Table 5. Appropriate restriction enzyme sequences were incorporated in the forward and reverse primers to subclone ORFs into the pcDNA 3.3 expression vector (Table 5). The reverse primer included the flag tag sequence and a stop codon. The flag tag - DYKDDDDK (where D=aspartic acid, Y=tyrosine and K=lysine) was used for western blotting detection and affinity purification (below). HEK-293A adherent cells were used for pilot expression, and the HEK-293 Freestyle (HEK-293F) cell line (Thermo Scientific, Wilmington, DE) was used for large-scale rTSP production in suspension cultures as described (Longo et al., 2013; Portolano et al., 2014).

Table 5 Primers for generation of mammalian expression constructs encoding pro-inflammation rTSPs

Protein ID	**PLASMID**	**PRIMER SEQUENCES (Restriction sites underlined)**
r*Aam*IGFBP-rP1	pcDNA3.3	For:5′-GGATCCTCGCAAGGAGTGCGGGCCTTG -3′ Rev:5′-GGATCCCTACTTATCGTCATCGTCCTTGTAGTCTTTTTT GGGCAGCACGTTGAGCTTGG-3′
r*Aam*IGFBP-rP6S	pcDNA3.3	For:5′- GGATCCTACGTCGGAACCGCACTGCG -3′ Rev: 5′-GGATCCCTACTTATCGTCATCGTCCTTGTAGTCTTTTTT CTCGTGATGGGCCGAGTCG-3′
r*Aam*IGFBP-rP6L	pcDNA3.3	For: 5′-GGATCCTACGTCGGAACCGCACTGCGAGG -3′ Rev: 5′-GGATCCCTACTTATCGTCATCGTCCTTGTAGTCCTCGTG GTGGGCCGAGTCGCCGCCG-3′
r*Aam*TCI	pcDNA3.3	For: 5′- TCAGATCTGAACGACTGCGTTTCCAACGGCTAC-3′ Rev:5′AGATCTCTACTTATCGTCATCGTCCTTGTAGTCTTTTTTGTT GAGCCACACACAGCACT-3′
*Aam*AAS8	pcDNA3.3	For: 5′- GGATCCTCAAGAGGAGCAAAAGGTGGCC-3′ Rev:5′GGATCCCTACTTATCGTCATCGTCCTTGTAGTCTTTTTTGAG GTGGTTCACTTGACCG-3′

r*Aam*IGFBP-rP1 = recombinant *Amblyomma americanum (Aam)* Insulin like growth factor binding protein-related protein 1, IGFBP-rP6S = Insulin like growth factor binding protein-related protein Short, IGFBP-rP6L = Insulin like growth factor binding protein- related protein Long, TCI = Tick carboxypeptidase inhibitor, AAS8 = Serine protease inhibitor. The linker (boldfaced and underlined) and flag tag (**AAAAAA**GACTACAAGGACGATGACGATAAG) were added to the reverse primers of IGFBP-rP1, IGFBP-rP6 S, TCI and AAS8. The *Bam*HI site was added to both reverse and forward primers of IGFBP-rP1, IGFBP-rP6S, IGFBP-rP6L and AAS8. The *Bgl*II site was added to both reverse and forward primers of TCI.

For pilot expression of rTSPs in mammalian cells, adherent 293A cells were grown to a monolayer of up to ~60-70% confluence in T-75 flasks containing 10 mL of Dulbecco's modified Eagle's medium (DMEM) supplemented with glutamine and heat-inactivated fetal bovine serum (10%) at 37°C with 5% carbon dioxide (CO_2) and 85-90% humidity. For transfection, 1 μg of plasmid DNA and 2.4 μL of the transfection reagent, polyethyleneamine (PEI) diluted in 180 μL of Opti-MEM (Thermo Scientific, Wilmington, DE) medium was incubated for 20 min at room temperature (RT). Following the incubation period, the 180 μL of the plasmid DNA-PEI mixture was added to each plate containing the cells, mixed gently by rocking the plate, and

incubated for ~48 h at 37°C in the humidified incubator. After 48 h, spent media was collected and used in an ELISA (enzyme linked immunosorbent assay) to check for rTSP expression using the antibody to flag tag-HRP (Horse-radish peroxidase) conjugated (Sigma-Aldrich, St. Louis, MO) and 3,3', 5,5' – tetramethylbenzidine substrate (Thermo Scientific, Wilmington, DE). The change in color was detected using an ELISA plate reader at 450 nm wavelength (Synergy H1, Biotek® microplate reader, BioTek Instruments, Inc., Winooski, VT, USA). The adherent cells were fixed by 100% ice cold methanol for 5 min at room temperature (RT). Subsequently, fixed cells were subjected to intracellular cell staining to detect rTSP expression using the antibody to 5 μg/mL flag tag conjugated with alkaline phosphatase enzyme (Sigma-Aldrich, St. Louis, MO) and substrate (Fast red TR salt, Naphthol, 10-50mM TRIS, pH-8, Sigma-Aldrich, St. Louis, MO).

For scaling up expression of rTSPs in mammalian cells, HEK-293F suspension cultures were grown to 1×10^6 viable cells/ml in 293 Freestyle medium (Thermo Scientific, Wilmington, DE) with shaking at 125 rpm in an incubator set to 8% CO_2 and 85% relative humidity. For transfection in suspension cultures, 30 μg recombinant plasmids and 30 μL of the transfection reagent, 293fectin (Thermo Scientific, Wilmington, DE) were incubated with 1 mL Opti-MEM media (Thermo Scientific, Wilmington, DE) separately. Following incubation, 293fectin and recombinant plasmids were combined and incubated for an additional 30 min and then added to 30 mL cell culture containing 30 million HEK-293F cells. The cell suspension was harvested after 72 h and cell viability was routinely confirmed by trypan blue staining (>90% viability).

After verification of rTSP expression using western blotting analysis, cell lysates and the supernatant were incubated with beads conjugated with the antibody to the FLAG tag (Sigma-Aldrich, St. Louis, MO) overnight at 4°C. The bound rTSP was eluted in 1 mL fractions using 0.1M Glycine buffer (pH 3.5) and neutralized with 25 μL 1M Tris-HCl (pH 8). Following verification of protein elution, fractions were combined and dialyzed against Tris-HCl buffer (50 mM Tris, 150mM NaCl, pH 7.4) using 10 kDa molecular cut off membrane spin filters (Pall Life Sciences ®, Port Washington, NY).

SDS-PAGE and western blotting analyses

Routine SDS-PAGE with Coomassie blue or silver staining and western blotting analysis was done to confirm purification. Proteins were resolved in 10%, 12.5% and 15% acrylamide/bis-acrylamide gels and stained with silver (Blum et al., 1966) and Coomassie stains (Blakesley and Boezi 1977). For western blotting analysis (Burnette 1981), resolved proteins were transferred to a PVDF (Polyvinylidene difluoride) membrane using the Thermo-fisher western blot transfer system (Thermo Scientific, Wilmington, DE). Following transfer, membranes were blocked overnight at 4°C in 5% skim milk in 1 X PBS-(0.05%)Tween20. Subsequently membranes were washed and then incubated with the antibody to the FLAG tag conjugated to HRP. The membranes were washed with 1 X PBS-Tween20 and the positive signal was detected using the chemiluminescent substrate (ECL^{TM} Prime Western blotting detection reagent, GE lifesciences, Pittsburg, PA) and X-ray films or using the Chemidoc MP gel doc (Biorad, Hercules, CA).

Macrophage cell activation assay

The Murine RAW 264.7 macrophage (MΦ) cell line was grown in Dulbecco's Modified Eagle's Medium (DMEM) (Lonza, Basel, Switzerland) that was supplemented with 4 mM L-glutamine, 4500 mg/L glucose, 1 mM sodium pyruvate, sodium bicarbonate, non-essential amino acid solution (Thermo Scientific, Wilmington, DE) and 10% fetal bovine serum (FBS) (Thermo Scientific, Wilmington, DE). Cells were maintained at 37°°C and 5% CO_2, 85-90% humidity incubator. MΦ were sub-cultured into 12 or 24 well plates overnight to 60-80% confluency. Routinely, the effects of rTSPs was investigated by co-culturing 1 x 10^6 MΦ cells with 0.10, 1.0, and 10 µg/mL of individual or cocktail mix of rTSPs. Following 24 h incubation, cultures were processed for the following assays: (A) flow cytometry of cell suspensions to measure expression of pro-inflammation co-stimulatory markers: CD40, CD80, and CD86, (B) Total Nitric Oxide detection kit (Pierce Total NO Assay kit, Thermo Scientific, Wilmington, DE) to determine nitric oxide (NO) in spent medium, (C) Cytokine ELISA assays to detect selected cytokines (TNFα, IL-1, IL-6, IL-10 and IL-12) in spent media, and (D) quantitative (q)RT-PCR to determine transcription of pro-inflammation and anti-inflammation markers, cytokines and co-stimulatory markers (primers in Table 6). The 24 h incubation was empirically determinedsince no or weak signals were detected at 8 and 12 h incubations. LPS (lipopolysaccharide), a bacterial cell wall component and a TLR4 (Toll like receptor) ligand (Kindly provided by Dr. Mwangi), were used as positive controls and media alone served as a negative control.

Flow cytometric analysis of pro-inflammation co-stimulatory markers (CD40, CD80, and CD86)

Treated and non-treated MΦ were detached using PBS-1mM EDTA, centrifuged and re-suspended in staining medium (DMEM with sodium azide). Immuno-labeling of murine cell surface markers was performed by incubating cells with fluorescein isothiocyanate (FITC) conjugated antibodies (15 μg/mL) to CD40 (Abcam, Cambridge, UK), CD86 (Thermo Scientific, Wilmington, DE) and CD80 (Thermo Scientific, Wilmington, DE) and isotype matched control mAbs IgG2a and IgG2b (Abcam, Cambridge, UK) for 30 min. After incubation, cells were washed three times with DMEM media containing 0.01% sodium azide and resuspended in 400 μL 1X PBS (pH-7.4) containing 1 μg/mL propidium iodide and analyzed by flow cytometry with parameters set to 10,000 events, filter setting 530/30 nm wavelength (BD FACS Caliber, San Jose, CA) using acquisition software BD CellQuest™ (BD Biosciences, San Jose, CA) and the analysis program FlowJo 9.8.5 (TreeStar, Ashland, OR) at digital imaging core facility, Department of Veterinary Pathobiology, CVM, TAMU.

Quantitative RT-PCR transcriptional analysis

Total RNA from the treated cells, positive and negative controls were isolated using TRizol (Thermo Scientific, Wilmington, DE). cDNA was synthesized following the manufacturer's protocol (Verso cDNA synthesis kit ®, Thermo Scientific, Wilmington, DE) using 500 ng total RNA. The qPCR (Quantitative polymerase chain reaction) was performed in a 50μl final reaction mix containing specific primers (Table 6), 1:10 diluted template cDNA, and 2X SYBR green PCR master mix (Thermo

Scientific, Wilmington, DE). All primers, forward and reverse were used at 300 nM concentration. The qPCR was performed in Applied Biosystems 7300 Real Time PCR System (Thermo Scientific, Wilmington, DE) and Biorad PCR machine (Biorad, Hercules, CA) set to the following cycling conditions thermal settings: 50^0C for 2 min for one cycle followed by 95^0C for 10 min, 95^0C for 40 cycles at 15 seconds interval and 60^0C for 1 minute. The mRNA expression levels were determined by delta delta Ct method (Livak and Schmittgen 2001). The GAPDH (glyceraldehyde 3-phosphate dehydrogenase) gene was used as an endogenous control. Amplifications from non-treated controls were used as a calibrator. The primers and concentration used in the study are listed in Table 6.

Table 6 Quantitative RT-PCR primers for pro/anti-inflammatory cytokine, co-stimulatory markers and signaling molecule expression

Pathway	TARGET	PRIMER SEQUENCE	References
Pro-inflammatory cytokines	TNF-α	For: 5′-ATGAGCACAGAAAGCATGA-3′ Rev: 5′-GAATGAGAAGAGGCTGAGA-3′	Dann et al., 2008
	IL-6	For: 5′-CTCTGGGAAATCGTGGAAAT-3′ Rev: 5′-CCAGTTTGGTAGCATCCATC-3′	Mazur et al., 2015
	IL-1	For:5′-CAACCAACAAGTGATATTCTCCATG-3′ Rev: 5′-′GATCCACACTCTCCAGCTGCA-3′	Guma et al., 2009
	IL-12	For: 5′-ATGGTGAAGACGGCCAGA-3′ Rev: 5′-CAGGTCTTCAATGTGCTGGTT-3′	
Anti-inflammtory cytokine	IL-10	For: 5′-GGGAAGACAATAACTGCACC-3′ ′ Rev: 5′-′GCTGGTCCTTTGTTTGAAAGA-3′	Vicente-Suarez et al., 2007
Co-stimulatory markers	CD40	For: 5′-GCTATGGGGCTGCTTGTTGA-3′ Rev: 5′-ATGGGTGGCATTGGGTCTTC-3′	Morgado et al., 2014
	CD80	For: 5′-CTGGGAAAAACCCCCAGAAG-3′ Rev: 5′-TGACAACGATGACGACGACTG-3′	Li et al., 2016
	CD86	For: 5′-CATGGGCTTGGCAATCCTTA-3′ Rev: 5′-AAATGGGCACGGCAGATATG-3′	Li et al., 2016
Signaling markers For the M1 Macrophage phenotype	NfKb-1	F: 5′-GAAATTCCTGATCCAGACAAAAAC-3′ R:5′ATCACTTCAATGGCCTCTGTGTAG-3′	Yamamoto et al., 2009
	NfKb-2	F:5′-CTGGTGGACACATACAGGAAGAC-3′ R:ATAGGCACTGTCTTCTTTCACCTC-3′	Yamamoto et al., 2009
	RelA	F:5′-CTTCCTCAGCCATGGTACCTCT-3′ R:5′CAAGTCTTCATCAGCATCAAACTG-3′	Yamamoto et al., 2009
	RelB	F:5′-CTTTGCCTATGATCCTTCTGC-3′ R: 5′-GAGTCCAGTGATAGGGGCTCT-3′	Yamamoto et al., 2009
	cFos	F:5′-GGGGACAGCCTTTCCTACTA-3′ R: 5′-CTGTCACCGTGGGGATAAAG-3′	Kim et al., 2013
	cJun	F:5′-ACGACCTTCTACGACGATGC-3′ R: 5′-CCAGGTTCAAGGTCATGCTC-3′	Kim et al., 2013
Internal control	GAPDH	For-5′-TATGTCGTGGAGTCTACTGGT-3′ Rev: 5′-GAGTTGTCATATTTCTCGT-3′	Davis et al., 2013

Cytokine analysis in the spent media

Cytokine specific ELISAs (Enzyme linked immunosorbent assay) were performed using Ready set go® eBioscience ELISA kits targeting IL-1, IL-6, IL-10, TGFβ and TNFα according to manufacturer's instructions (eBioscience®, Thermo Scientific, Wilmington, DE). Capture antibodies in 1 X coating buffer (dilution 1:250)

specific for IL-1, IL-6, IL-10, TGFβ and TNF-α were coated separately on ELISA plates (Corning Coster® 9018, Corning, NY) with volume of 100 μL in each well in triplicates overnight at 4^0C. On the following day, the plates were washed 3 times with 250 μL wash buffer (1X PBS, 0.05% Tween 20) and blocked with ELISA diluent (eBioscience®, Thermo Scientific, Wilmington, DE) for 1 h at RT. Subsequently spent media from MΦ cultures, 100 μL/well were added to the wells in triplicate and incubated for 2 h RT for binding. After incubation, the wells were washed three times and 100 μL of detection antibody (1:250 dilution) in 1X ELISA diluent buffer. The plates were sealed and incubated for 1 h at RT. After 1 h, the plates were washed and 100 μL of avidin-HRP (1:25 dilution) was added and incubated for 30 min at RT. The plates were washed and 1X TMB (tetramethylbenzidine) was added to each well for enzymatic colorimetric reaction. The reaction was stopped with 50 μL stop solution (2N H_2SO_4). Optical densities were measured using an ELISA plate reader at 450 nm wavelength (Synergy H1, Biotek® microplate reader, BioTek Instruments, Inc., Winooski, VT, USA).

Nitric oxide detection

Nitric oxide metabolites released in the cell culture supernatant as nitrate or nitrite was detected by Total Nitric Oxide detection kit (Pierce Total NO Assay kit, Thermo Scientific, Wilmington, DE) according to the manufacturer's protocol. Nitrate reductase enzyme and cofactor NADPH (Nicotinamide adenine dinucleotide phosphate) were added to the wells including the positive and negative control to convert nitrate to nitrite in the media. Finally, Griess reagent I (Sulfanilamide) and Griess reagent II (N-(1-

Naphthyl) Ethylenediamine) was added to convert the nitrite to an Azo compound that is purple in color. Photometric measurement of the absorbance due to this azo chromophore determined the NO_2^- (nitrite) concentration at 540 nm wavelength using an ELISA plate reader (Synergy H1, Biotek® microplate reader, BioTek Instruments, Inc., Winooski, VT, USA).

Cell staining

Based on detection of pro-inflammation co-stimulatory markers and cytokines, the pro-inflammation effects of the five rTSPs (*Aam*IGFBP-rP1, *Aam*IGFBP-rP6L, *Aam*IGFBP-rP6S, AAS8, and *Aam*TCI) on RAW 264.7 MΦ were similar to the effects of LPS. To investigate similarities or differences in physical phenotypes of LPS and PI-rTSP (*Aam*IGFBP-rP1, *Aam*IGFBP-rP6L, and *Aam*IGFBP-rP6S) activated MΦ were seeded on chambered slides and immuno-stained with the antibody to Actin (Abcam, Cambridge, UK). Following 24 h treatment, cells were fixed with ice cold methanol, washed with 1X PBS and blocked with 1% BSA, 22.52 mg/ml glycine in PBST (PBS+0.1% Tween 20) for 30 min. Cells were incubated with the anti-actin antibody overnight at 4ºC. The following day, the cells were washed in 1 X PBS and incubated with secondary antibody conjugated with Alexa Fluor (Abcam, Cambridge, UK) for 1 h in the dark. The cells were washed, treated with mounting media containing DAPI (Thermo Scientific, Wilmington, DE) for nuclei staining and cell staining was analyzed by confocal microscopy. Confocal images were acquired with Ziess LSM 780 confocal microscope and merged using Zen 2012 SP1 (black edition) software in the Imaging facility at the CVM TAMU.

Statistical analysis

Data acquisition for flow cytometry was performed by using BD Cell Quest™ (BD Bioscience, San Jose, CA). The data analysis program used was FlowJo 9.8.5 (TreeStar, Ashland, OR). The cells with >90% viability was selected by gating on the flow cytometer. One Way ANOVA analysis and Dunnett's post Hoc analyses of median fluorescent intensities and absorbance values for cytokine and nitric secretion were done using PRISM software (GraphPad Software, San Diego, CA, USA) to determine statistical differences between the control and the treatments. The statistical significance of mRNA transcript abundance was determined for each replicate using the formula $2^{-\Delta\Delta Ct}$ as described (Livak and Schmittgen, 2001).

Results

Amblyomma americanum secretes pro- and anti-inflammatory proteins in its saliva

Ticks accomplish feeding and transmission of tick borne disease (TBD) agents by injecting multiple tick saliva proteins (TSP) into the host to evade host defense. Prior to this dissertation research, our lab identified *I. scapularis* (Lewis et al., 2015, Kim et al., 2016) and *A. americanum* (Radulovic et al., 2014, Kim et al., *in submission*) TSPs that are sequentially injected into animals during tick feeding. Some of these TSPs were already expressed as recombinant (r) proteins in yeast and insect cells (Mulenga et al., 2013, Kim et al., 2015, Radulovic et al., 2014, Ibelli et al., 2014, Kim et al., *in preparation*) (Table 7). To begin characterizing their role(s) in tick feeding regulation, flow cytometry was successfully used to screen the effects of 15 selected rTSP; *A. americanum* (n=12) and *I. scapularis* (n=3) on RAW 267.4 MΦ expression of pro-

inflammatory co-stimulatory markers (CD40, CD80, and CD86) and nitric oxide (NO) secretion using the Griess assay (Griess 1879). Lipopolysaccharide (LPS), a structural component of gram-negative bacteria and a toll-like receptor 4 (TLR4) ligand activator of MΦ (Janssens and Beyaert 2003) and media only were used as positive and negative controls. This preliminary analysis revealed that, similar to LPS, adding various doses of five of the 15 tested rTSPs, *A. americanum* (*Aam*) insulin-like growth factor binding protein-related proteins [*Aam*IGFBP-rP1, 0.0033 nM, 0.033 nM and 0.33 nM, *Aam*IGFBP-rP6 short (S), 0.0045 nM, 0.045 nM, 0.45 nM *Aam*IGFBP-rP6 long (L), 0.0041 nM, 0.041 nM, 0.41 nM, *A. americanum* serine protease inhibitor (AAS) 8, 0.0024 nM, 0.024 nM, 0.24 nM and tick carboxypeptidase inhibitor (TCI), 0.01 nM, 0.10 nM, 1 nM] stimulated MΦ to express CD40, CD80, and CD86 and secrete NO significantly above the media negative control (Table 7). In contrast, two rTSPs, *A. americanum* serpins, rAAS27 and rAAS41 (Porter et al., 2015, Kim et al., *in preparation,* Tirloni et al., *in preparation*) suppressed the expression of pro-inflammation co-stimulatory markers and NO secretion below negative control levels (Table 7). Five of the 15 rTSPs (asterisks marked in Table 7) that did not stimulate MΦ as there was less or no apparent difference from media control and were removed from further investigation. Furthermore, *A. americanum* histamine release factor (r*Aam*HRF) and histamine binding proteins (AV52) that stimulated MΦ expression of the three co-stimulatory markers at the highest dose but not at lower doses were not further investigated. Here after, the five *A. americanum* rTSPs for which all three dosages stimulated MΦ to express the three pro-inflammation co-stimulatory markers and

secretion of NO significantly above background are referred to as pro-inflammatory (PI), and those that suppressed as anti-inflammatory (AI).

Table 7 Preliminary analysis showing effect of *Ixodes scapularis* (Ixsc) and *Amblyomma americanum* (*Aam* and AAS) rTSPs on RAW macrophages

Protein ID	**PC (µg/ mL)**	**PFI**			**Percent NO level**	**EL (EU/mL)**	**Tick species**
		CD40	**CD80**	**CD86**			
r*Aam*IGFBP-rP1^{+}	0.1	26.20	27.47	14.43	6.88	0.05	*Amblyomma americanum*
	1	28.60	20.73	14.43	0		
	10	25.50	10.30	10.45	0		
r*Aam*IGFBP-rP6L^{+}	0.1	26.23	19.68	16.49	3.94	0.27	*A. americanum*
	1	26.23	11.36	18.70	0		
	10	21.78	18.62	13.40	0		
r*Aam*IGFBP-rP6S^{+}	0.1	23.30	11.36	14.43	0	0.005	*A. americanum*
	1	26.90	21.79	18.70	0		
	10	23.10	19.68	16.49	0		
rAAS8^{+}	0.1	28.10	16.51	10.45	1.26	0.18	*A. americanum*
	1	27.90	20.73	14.43	0		
	10	22.90	10.30	8.54	0		
r*Am*TCI	0.1	20.99	0	13.25	14.33	0.01	*A. americanum*
	1	50.65	6.62	26.25	23.73		
	10	53.02	75.49	28.50	17.82		
AV42*	0.1	17.55	0	13.25	19.05	0.037	*A. americanum*
	1	28.11	0	15.25	23.88		
	10	10.43	0	10.25	14.97		
AV52*	0.1	0	0	0	3.72	Not done	*A. americanum*
	1	0	0	0	3.06		
	10	7.40	51.10	30.80	10.95		
*Aam*CRT*	0.1	3.67	0	4.50	16.84	0.08	*A. americanum*
	1	28.11	0	0	14.86		
	10	41.16	1.82	32.00	20.10		
r*Aam*HRF*	0.1	0	0	0	1.20	0.07	*A. americanum*
	1	0	0	0	0		
	10	14.60	26.20	17.50	9.41		
rAAS19*	0.1	0	0	0	1.42	0.01	*A. americanum*
	1	0.90	0	1.70	4.49		
	10	0	0	1.70	7.55		
IxscS8	0.1	0	0	0	Not done	0.02	*Ixodes scapularis*
	1	0	0	0	Not done		
	10	0	0	0	Not done		
IxscS4	0.1	0	0	0	Not done	0.28	*I. scapularis*
	1	0	0	0	Not done		
	10	0	0	0	Not done		
rAAS27	0.1	0	22.41 (-)	0	Not done	0.00	*A. americanum*

Table 7 Continued

Protein ID	PC (µg/ mL)	PFI			Percent NO level	EL (EU/mL)	Tick species
		CD40	CD80	CD86			
	1	1.29 (-)	24.10 (-)	3.15	Not done		
	10	2.33 (-)	26.13 (-)	7.72	Not done		
rAAS41	0.1	0	24.10 (-)	0	Not done	0.0098	*A. americanum*
	1	9.52 (-)	9.04 (-)	0	Not done		
	10	0	14.04 (-)	0	Not done		
IxscTCI*	0.1	7.29	7.10	Not done	9.45	0.0136	*I. scapularis*
	1	0	5.59	Not done	22.80		
	10	3.38	7.02	Not done	23.79		

PC=Protein concentration, PFI=Percent fluorescent intensity, NO = Nitric oxide, EL= Endotoxin level, EU= Endotoxin unit, Plus (+) sign= Insect cell expressed recombinant proteins, Tilde (~) sign = Yeast expressed recombinant proteins, Asterisks (*) sign = Removed from further analysis, (-) = Down-regulated, zero (0) = not different from background. AAS = *A. americanum* serine protease inhibitor (serpin), IxscS = *I. scapularis* serpin. *Aam*TCI and IxscTCI = *A. americanum* and *I. scapularis* tick carboxypeptidase inhibitor.

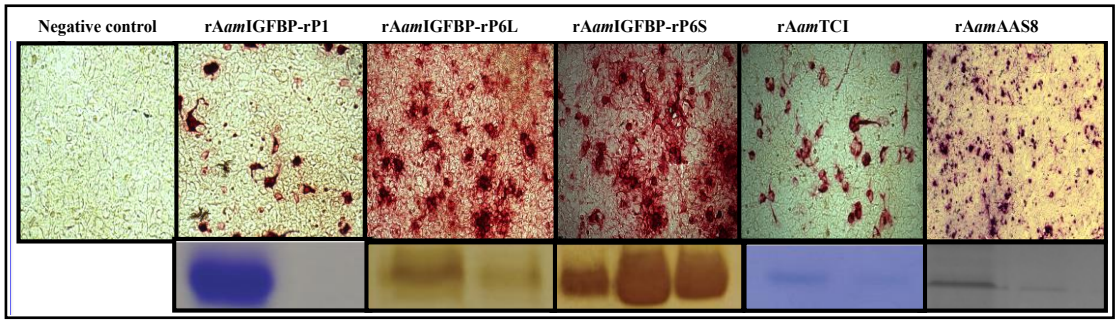


Figure 1. Expression and affinity purification of *Amblyomma americanum* (*Aam*) pro-inflammation recombinant tick saliva proteins (rTSPs) in human embryonic kidney (HEK) 293 cells. Mature protein coding proteins were sub-cloned into modified pcDNA plasmids and transfected into HEK cells as described in materials and methods. **Top panel:** HEK cells expressing recombinant tick saliva proteins were immuno-stained using the antibody to the FLAG tag. **Bottom panel:** Affinity purified rTSPs were subjected to SDS-PAGE electrophoresis on 12.5 or 15% acrylamide gels and stained with Coommassie or silver stains. Left to right: Negative control, r*Aam*IGFBP-rP1 (*A. americanum* insulin like growth factor binding protein-related protein 1), resolved at approximately 30 kD, r*Aam*IGFBP-rP6S (*A. americanum* insulin like growth factor binding protein-related protein 1), resolved at approx. 24 kD, r*Aam*IGFBP-rP6L (*A. americanum* insulin like growth factor long), resolved at approx. 24 kD, r*Aam*TCI (*A. americanum* tick carboxypeptidase inhibitor), resolved at approximately 10 kD, r*Aam*S8 (*A. americanum* Serine protease inhibitor 8), resolved at approx. 47 kD.

Mammalian cell expressed PI-rTSPs stimulated MΦ to express pro-inflammation co-stimulatory markers (CD40, CD80, and CD86) and cytokines

To rule out involvement of endotoxin contamination in the observed stimulation of MΦ by insect and yeast cell expressed PI-rTSPs (Table 7), the five PI-rTSPs (r*Aam*IGFBP-rP1, r*Aam*IGFBP-rP6S, r*Aam*IGFBP-rP6L, rAAS8, and r*Aam*TCI) were re-expressed in human embryonic kidney (HEK)-293 expression system (Fig.1). When co-cultured with MΦ for 24 h, all five-mammalian cell expressed PI-rTSPs stimulated MΦ to express CD40 significantly above media with p-values of below 0.05 (single asterisks [*]), 0.01 (double asterisks [**]), and 0.001 (triple asterisks [***]), respectively (Figs.2A). Fig.2B shows that CD80 was significantly induced in MΦ that were treated

with 0.0033 and 0.033 nM, but not 0.33 nM of r*Aam*IGFBP-rP1, 0.0041 and 0.041, but not 0.41 nM of r*Aam*IGFBP-rP6L, 0.24 but not 0.0024 and 0.024 nM of rAAS8, while expression in r*Aam*TCI treated cells was minimal to negligible. In the case of CD86, except for 0.41 nM r*Aam*IGFBP-rP6L and 0.45 nM r*Aam*IGFBP-rP6S treated for which expression was significantly above medium control, all other treatments were insignificant (Fig.2C). Notable in Fig. 2 was that the lowest doses of r*Aam*IGFBP-rP1 and r*Aam*IGFBP-rP6S significantly induced highest expression of CD40, and CD80 for the former (Fig.2A, 2B), while CD86 expression was observed at higher doses of all five rTSPs (Fig. 2C). Although treatment of MΦ with PI-rTSPs induced nitric oxide above medium control, it was not statistically significant (Fig.2D)

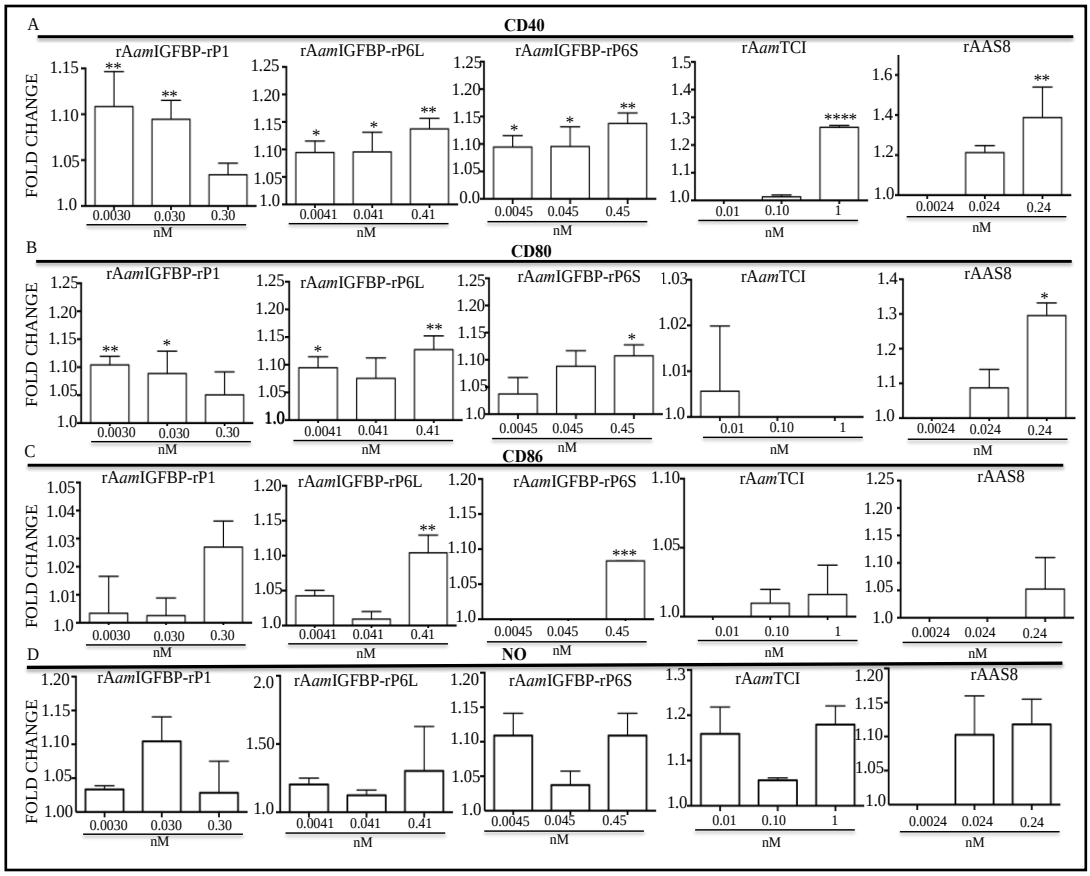


Figure 2. Mammalian cell expressed pro-inflammation (PI)-rTSPs stimulate MΦ to express pro-inflammation co-stimulatory markers and nitric oxide (NO). RAW macrophages were co-cultured with r*Am*IGFBP-rP1 (0.0030 nM, 0.030 nM, 0.30 nM), r*Am*IGFBP-rP6L (0.0041 nM, 0.041 nM, 0.41 nM), r*Am*IGFBP-rP6S (0.0045 nM, 0.045 nM, 0.45 nM), rAAS8 (0.0024 nM, 0.024 nM, 0.24 nM), r*Aam*TCI (0.01 nM, 0.10 nM, 1 nM) rTSPs for 24 h. LPS was used positive. Data is represented as fold change of treatments above negative control (Treatment median fluorescent intensity (MFI)/negative control MFI for cell surface marker detection and Treatment absorbance/negative control absorbance for NO detection). Data are reported as the mean (three biological replicates) ± SE of three replicates. (*) = $p \leq 0.05$, (**) = $p \leq 0.01$, (***) = $p \leq 0.001$, (****) = $p \leq 0.0001$, indicating statistically significant difference between media and treatments using one way ANOVA followed by post hoc Dunnett's multiple comparisons test.

Motivated by the fact that activated MΦ that were expressed pro-inflammation co-stimulator markers are characterized by secretion of a range of cytokines (Duque and Descoteaux 2014), quantitative (q) RT-PCR (primers in Table 6) and cytokine ELISA were conducted to determine effects of PI-rTSP on MΦ expression of pro-inflammatory

cytokines, IL-1, IL-6, IL-12, and TNF-α, and the anti-inflammatory cytokine, IL-10 (Fig. 3). Quantitative RT-PCR revealed that the TNFα transcript was expressed significantly above background in MΦ that were treated with 0.033 nM r*Aam*IGFBP-rP1, 0.41 nM r*Aam*IGFBP-rP6L, and all three doses of r*Aam*TCI (Fig.3A). Similarly the IL-6 transcript was expressed above background in MΦ that were treated with 0.033 nM r*Aam*IGFBP-rP1 and 0.041 nM r*Aam*IGFBP-rP6L, but not in other treatments (Fig.3B). Likewise cytokine ELISA revealed that TNF-α, but not other cytokines were secreted at levels there were significantly above media control (Figs.3C). As shown in Fig.3C, TNF-α was secreted in significant amounts by MΦ that were treated with 0.33 nM r*Aam*IGFBP-rP1 ($p<0.0001$), 0.041 nM r*Aam*IGFBP-rP6L ($p<0.0001$), 0.024 nM ($p<0.05$) and 0.24 nM ($p<0.0001$) rAAS8. In the case of IL-6, except for MΦ that were treated with 0.24 nM ($p<0.001$) rAAS8, levels in other treatments were apparently higher or insignificant (Fig.3D). It is interesting to note that the highest expression levels of TNF-α secretion was in MΦ that were co-incubated with low doses of r*Aam*IGFBP-rP6L (0.041 μM) and the highest dose of r*Aam*IGFBP-rP1 (Fig. 3C). Given the low expression levels of rAAS8 and r*Aam*TCI in mammalian cells (Fig.1), further analysis of these two proteins was not pursued.

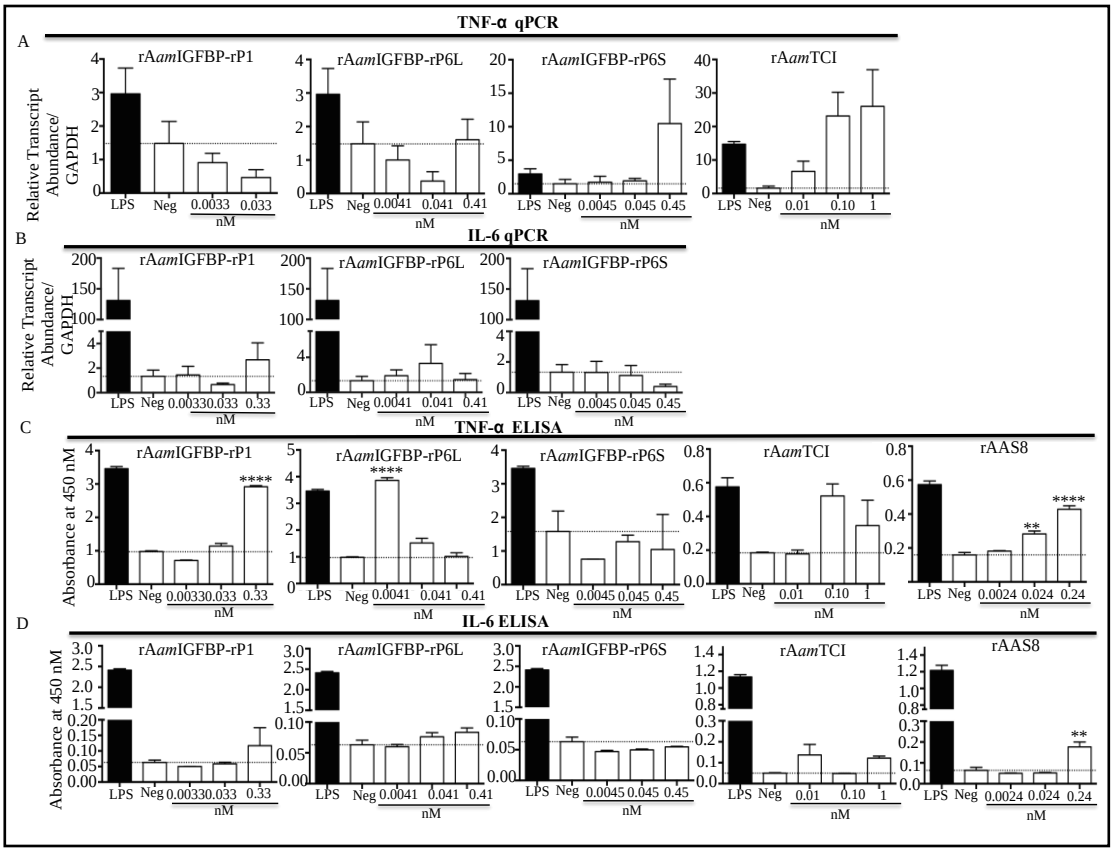


Figure 3. Mammalian cell expressed pro-inflammation (PI)-rTSPs stimulate MΦ to express pro-inflammation cytokines. RAW macrophages were co-cultured with r*Am*IGFBP-rP1, r*Am*IGFBP-rP6L, r*Am*IGFBP-rP6S, rAAS8, r*Am*TCI rTSPs for 24 h. LPS was used positive control and culture media only as negative control. Cells and spent media were harvested and respectively subjected to quantitative expression analysis of PI cytokines TNFα and IL-6 (Fig.3A, B), GAPDH was used as internal reference and Media only was used as calibrator, ELISA to detect secretion of PI cytokines (Fig.3C, D) as described. Concentrations r*Am*IGFBP-rP1 (0.0030 nM, 0.030 nM, 0.30 nM), r*Am*IGFBP-rP6L (0.0041 nM, 0.041 nM, 0.41 nM), r*Am*IGFBP-rP6S (0.0045 nM, 0.045 nM, 0.45 nM), rAAS8 (0.0024 nM, 0.024 nM, 0.24 nM), r*Aam*TCI (0.01 nM, 0.10 nM, 1 nM) rTSPs were used. Data are reported as the mean (three biological replicates) ± SE of three replicates. (*) = $p \leq 0.05$, (**) = $p \leq 0.01$, (***) = $p \leq 0.001$, (****) = $p \leq 0.0001$, indicating statistically significant difference between media and treatments using one way ANOVA followed by post hoc Dunnett's multiple comparisons test.

As *Aam*IGFBP-rP1, r*Aam*IGFBP-rP6S, and r*Aam*IGFBP-rP6L are structurally (Mulenga et al., 2010) and functionally similar as insulin binding proteins (Radulovic et al., 2014, Radulovic et al., *in preparation* for *Aam*IGFBP-rP6S and *Aam*IGFBP-rP6L) and the fact that all three proteins have similar secretion kinetics during *A. americanum* tick feeding (Kim et al., *in preparation*), I proceeded to analyze the effects of these proteins as a cocktail mix to investigate the possibility for synergy. It was interesting to note that the cocktail of the three PI-rTSPs (*Aam*IGFBP-rP1, *Aam*IGFBP-rP6S, and *Aam*IGFBP-rP6L) were synergistic in that secretion levels of TNF-α, IL-1, and IL-6 were similar to those detected in spent media of LPS treated MΦ (Fig. 4A-C). Interestingly, detectable amounts of anti-inflammation cytokines IL-10 and TGFβ were also present in spent media of these MΦ (Fig. 4D, E). Given similarities in cytokine expression levels between LPS and PI-rTSP activated MΦ, the potential for any similarities in physical attributes was investigated through staining. Staining with the Alexa Fluor labeled antibody to actin revealed that the physical phenotypes of LPS and PI-rTSPs activated MΦ were comparable as indicated by spindle formation (Fig.4F).

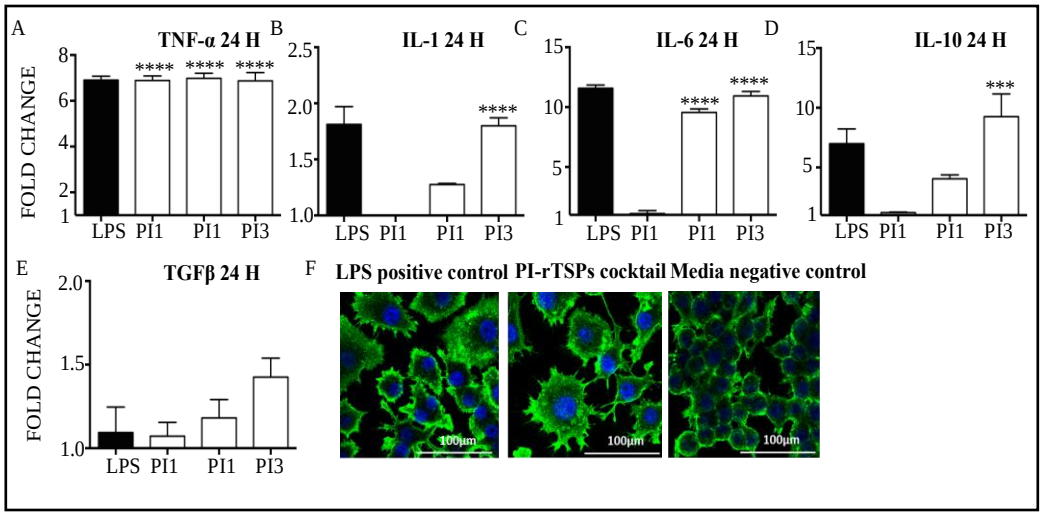


Figure 4. The cocktail mix of pro-inflammation (PI) rTSPs: r*Aam*IGFBP-rP1, r*Aam*IGFBP-rP6S, and r*Aam*IGFBP-rPL6L synergistically stimulated MΦ to express high levels of pro-inflammation cytokines and anti-inflammation cytokines. RAW macrophages were treated with PI-rTSPs as described. (Fig4A-4E) supernatants from the treatments were collected following 24 h incubation for detection of cytokine TNFα, IL-1, IL-6, IL-10 and TGFβ using cytokine ELISA kits. Data are reported as the mean (three biological replicates) ± SE of three replicates and represented as fold change of treatments above negative control (Treatment absorbance/negative control absorbance). (*) = $p \leq 0.05$, (**) = $p \leq 0.01$, (***) = $p \leq 0.001$, (****) = $p \leq 0.0001$, indicating statistically significant difference between media and treatments using one way ANOVA followed by post hoc Dunnett's multiple comparisons test. (Fig.4F) MΦ that were stimulated with PI-rTSPs were fixed with 100% methanol, incubated with 1% BSA, 22.52 mg/mL glycine PBST (PBS-Tween) blocking solution for 1 h. The cells were incubated overnight at 4º C with the primary antibody (Ab), goat anti-mouse actin antibody. Following appropriate washing of non-specifically bound Ab, MΦ were incubated with the secondary antibody conjugated with Alexa fluor for 30 min at room temperature. Nuclear DNA was labeled with DAPI (shown in blue). Images were captured using confocal microscope. PI1, PI2, and PI3 = 0.1, 1.0, and 10 µg of the PI-rTSP cocktail.

Co-incubating LPS and PI-rTSP activated MΦ with rAAS27 and rAAS41 reverses expression of pro-inflammation markers

In preliminary studies, the expression levels of co-stimulatory molecules; CD40, CD80, and CD86 were detected below background in MΦ treated with either rAAS27 or rAAS41 (Table 7). This suggested two possibilities: either that the two serpins (i) prevented activation of, or (ii) selectively affected functions of activated MΦ. The first

possibility was ruled out in that co-incubating MΦ with rAAS27 or rAAS41 individually or as a cocktail did not impact expression levels of co-stimulatory markers or cytokines (not shown). To investigate the second possibility, MΦ were first activated with LPS (100 ng) or various dosages (0.1, 1, 10 μg) of r*Aam*IGFBP-rP1, or r*Aam*IGFBP-rP6L, and r*Aam*IGFBP-rP6S (PI) for 24 h and then replacing culture media with fresh media that contained various dosages (0.1, 1, 10 μg) of rAAS27 and rAAS41 or the cocktail of both proteins for an additional 24 h. Individually, rAAS27 or rAAS41 had modest effects on expression of co-stimulatory markers in LPS activated MΦ (not shown). However, the rAAS27 and rAAS41 cocktail had a much more dramatic effect on LPS activated MΦ as analyzed by flow cytometry using staining for the various CD markers (Fig.5). Adding various dosages of the rAAS27 and rAAS41 cocktail on MΦ that were activated with LPS, reduced the expression levels of CD40, CD80 and CD86 by 43-48%, 16-28%, and 10-13% respectively (Fig.5A and inserts). Likewise, albeit at low levels, co-incubating PI-rTSPs activated MΦ with the rAAS27 and AAS41 cocktail respectively reduced the expression of CD40 and CD80 by ~6-8.5% and ~12-13%, while CD86 expression by less than 3% (Fig.5B and inserts).

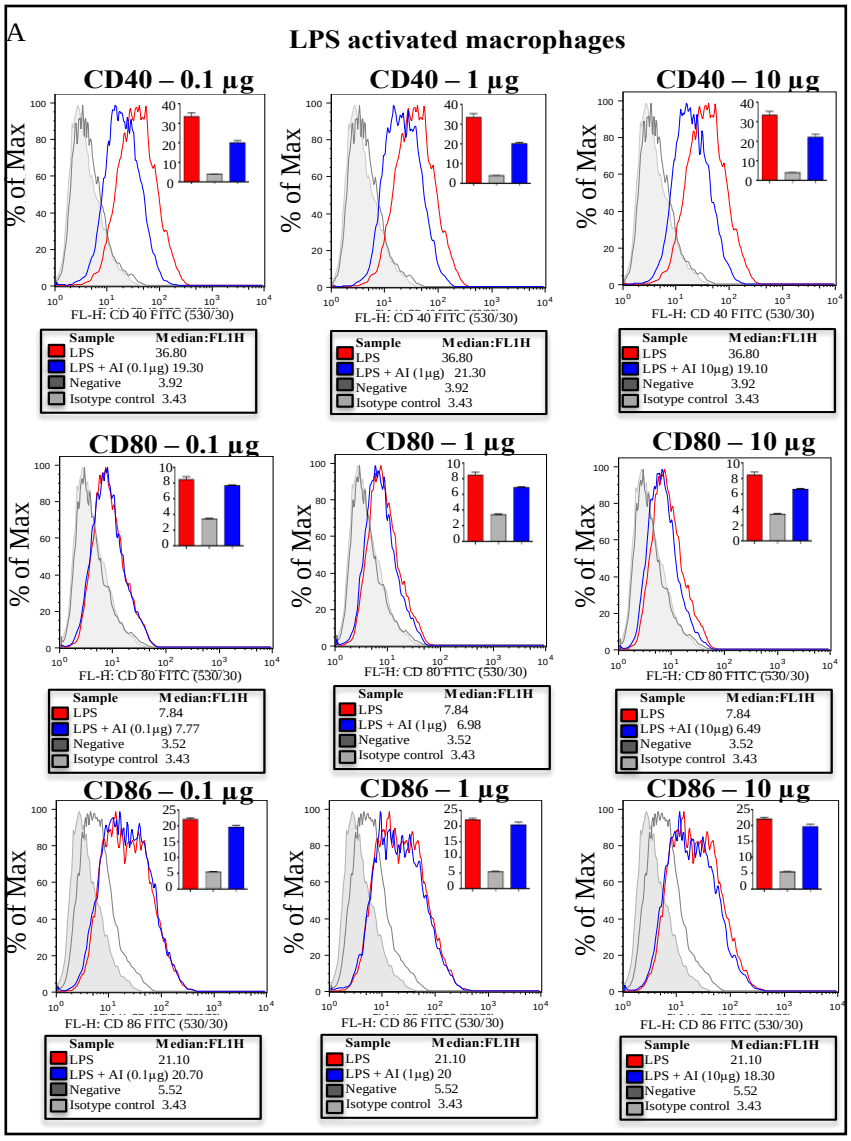
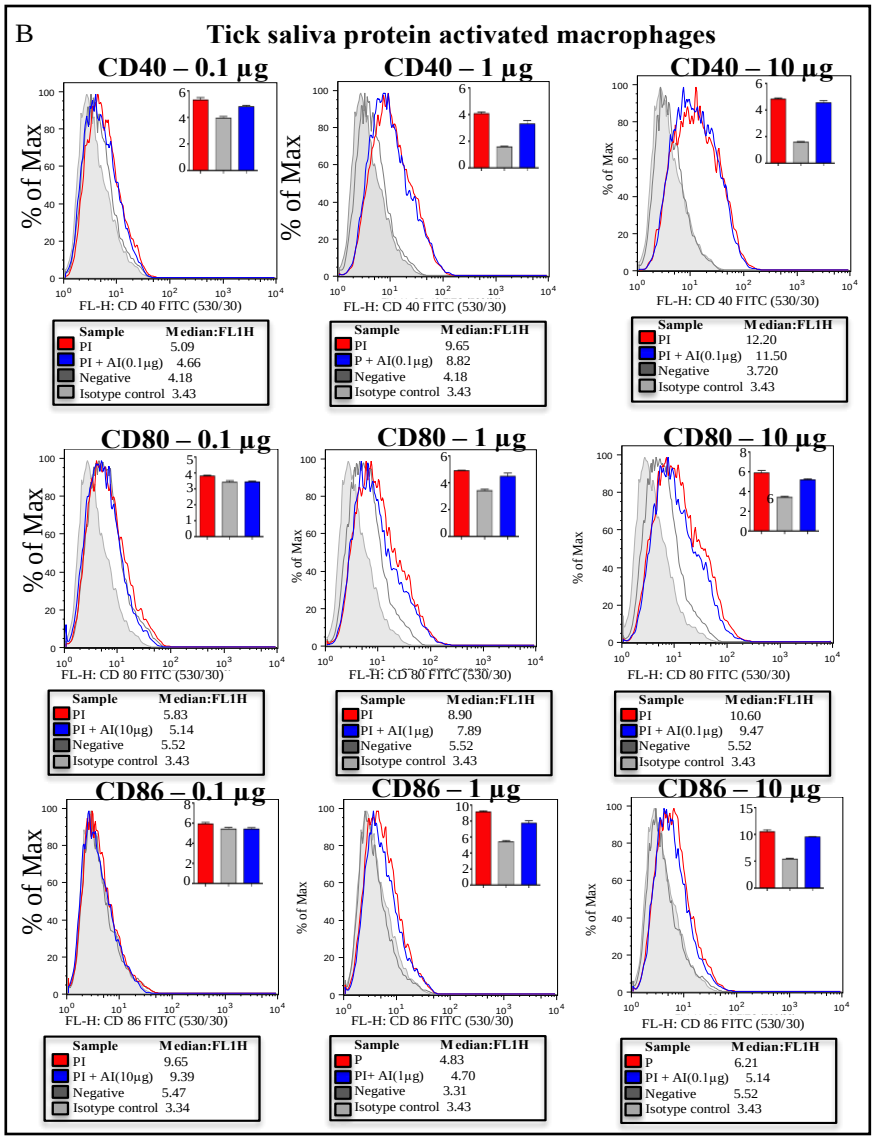


Figure 5. *Amblyomma americanum* anti-inflammation (AI)-rTSP reverses the expression of costimulatory markers by LPS and *A. americanum* pro-inflammation (PI)-rTSP activated macrophages. RAW macrophages (MΦ) were cultured in the presence of LPS (Fig.5A) or with various doses of PI-rTSPs as noted (Fig.5B) for 24 h. Subsequently culture media was replaced with media that contained various doses of AI-rTSPs as noted for an additional 24 h for both LPS and PI-rTSP activated cells. Subsequently cells were harvested and immuno-stained with FITC conjugated antibody to CD40, CD80 and CD86 and analyzed by Flow cytometry. Filled gray chromatogram = isotype control, dark gray line chromatogram and bar graph = media treated cells, blue line chromatograms and bar graphs = LPS or PI-rTSP pre-activated MΦ co-incubated with AI-rTSPs, red chromatogram and bar graph = LPS or PI-rTSP activated MΦ only. Bar graph inserts show mean median fluorescent intensity in MΦ that were treated with LPS

Figure 5 Continued



While individual rAAS41 and rAAS27 did not affect the expression of co-stimulatory markers by LPS activated MΦ (not shown), it was notable that adding 0.024 and 0.24 nM rAAS27 and rAAS41 to LPS activated MΦ respectively reduced transcript abundance of pro-inflammation cytokines, TNFα and IL-1 by 7-11% and 12-33% but not IL6 (Fig. 6A, B). Likewise, spent media of these MΦ contained reduced amounts of TNFα, IL1, and IL6 (Figs.6C, D, E). We observed comparable results when LPS activated MΦ were co-incubated with rAAS41 for which we determined that transcript abundance of TNFα and IL-1 and not IL-6 were suppressed respectively (Fig. 6F-G).

Cytokine ELISA revealed that rAAS41 treatment of LPS activated MΦ reduced secretion of TNFα, IL-1 and IL-6 respectively (Fig.6H, I, J). On the other hand, co-incubating the AI-rTSP cocktail with MΦ that were pre-activated with PI-rTSP did not affect cytokine transcription (not shown). Fig.7 demonstrates that the effects of the AI-rTSPs cocktail on LPS and PI-rTSPs activated MΦ secretion of cytokines was mixed. Except for TNFα, which was dose dependently suppressed (Fig.7A T1), AI-rTSPs treatment did not affect secretion levels of cytokines MΦ that were pre-activated with LPS (Fig.7B-7E, T1).

Similarly, AI-rTSP treatment of MΦ that were pre-activated with PI-rTSP did not affect cytokine secretion of TNFα, IL1, IL6, and IL-10 (Figs.7B-7D, T3 vs T4). It is notable that whereas adding AI-rTSP cocktail did not have any effects on TGFβ secretion in LPS activated MΦ, it was interesting to note that this treatment significantly induced secretion of TGFβ in MΦ that were pre-activated with PI-rTSPs (Fig7E, T3 vs T4).

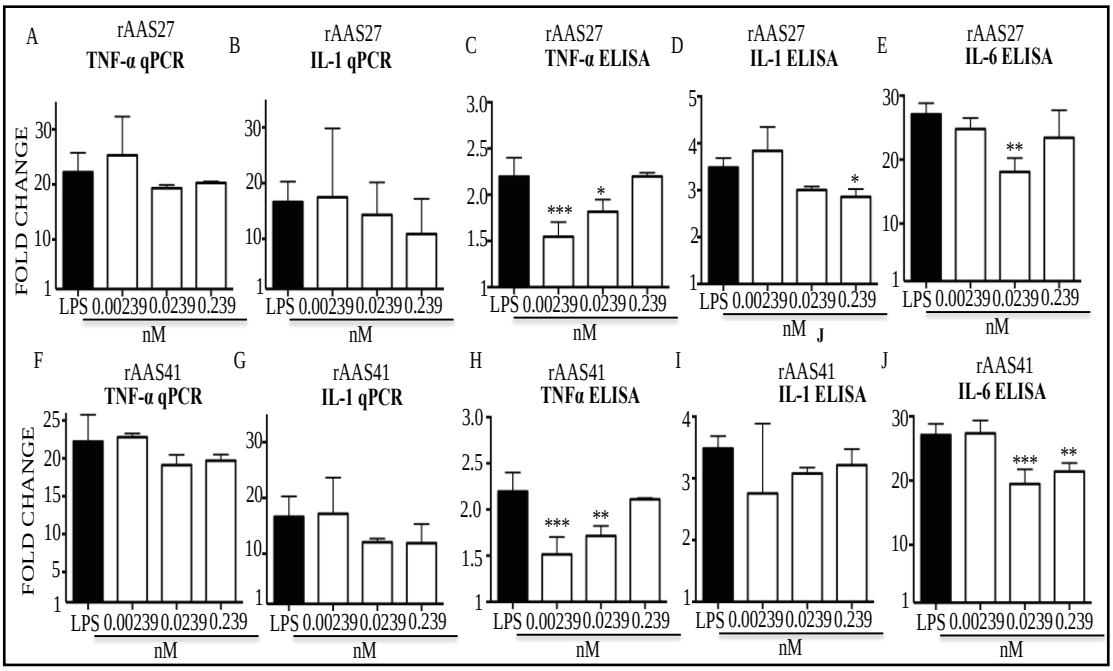


Figure 6. ***Amblyomma americanum*** **anti-inflammation (AI)-rTSP, rAAS27 and rAAS41 reverses the expression of pro-inflammation cytokines by LPS activated macrophages.** RAW macrophages (MΦ) were cultured in the presence of LPS for 24 h. Subsequently culture media was replaced with media that contained various doses of AI-rTSPs as noted for an additional 24 h. Cells and spent media in three biological replicates were processed to respectively determine transcription and secretion levels of cytokines TNFα, IL1 and IL6 using ELISA and qRT-PCR, GAPDH was used as internal reference and Media only was used as calibrator. rAAS27 concentrations, 0.00239, 0.0239, and 0.239 nM, rAAS41 concentrations, 0.00239, 0.0239, and 0.239 nM were used respectively. Data is represented as fold change: Treatment absorbance values/media control absorbance values and reported as the mean (three biological replicates) ± SE of three replicates. (*) = $p \leq 0.05$, (**) = $p \leq 0.01$, (***) = $p \leq 0.001$, (****) = $p \leq 0.0001$, indicating statistically significant difference between media and treatments using one way ANOVA followed by post hoc Dunnett's multiple comparisons test.

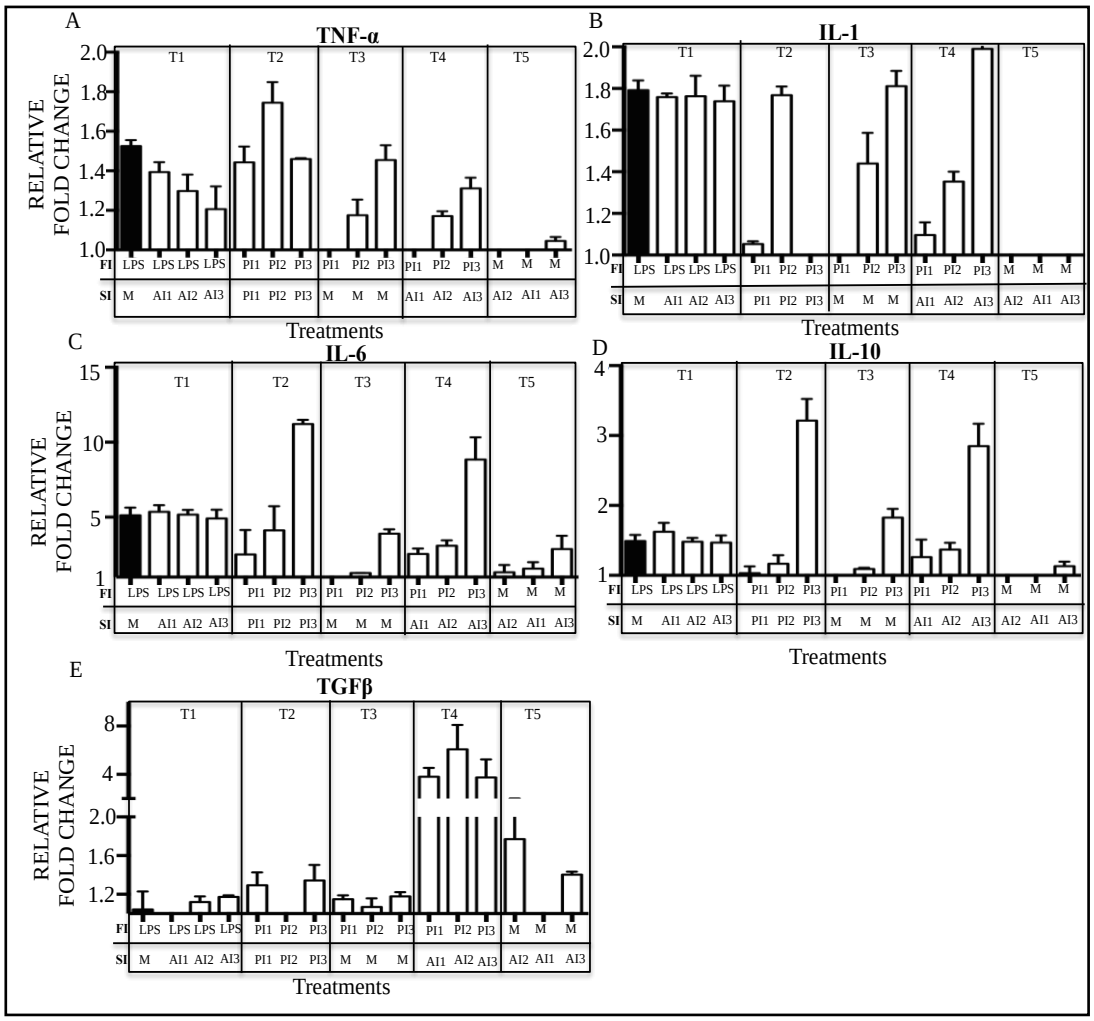


Figure 7. ***Amblyomma americanum*** **anti-inflammation (AI)-rTSP, rAAS27 and rAAS41 cocktail mix reverses TNFα and enhances TGFβ expression, but did not affect expression of other pro-inflammation cytokines in macrophages that were pre-activated with LPS or the PI-rTSP cocktail (r*Aam*IGFBP-rP1, r*Aam*IGFBP-rP6S, and r*Aam*IGFBP-rPL6).** RAW macrophages were cultured for 24 h with 100 ng/μL LPS (marked as T1) or various doses of the PI-rTSP cocktail (T2-T4). The secretion levels of cytokines; TNF-α, IL-1, IL 6, TGFβ and IL-10 in spent media was determined by ELISA. Data are reported as the mean (three biological replicates) ± SE of three replicates and represented as fold change of treatments above negative control (Treatment absorbance/negative control absorbance). FI = cells incubated for 24 h with indicated treatments. SI = remove 24 h spent media, and continue to incubate with fresh media containing indicated reagents. LPS = 100 ng LPS, M = Media only, PI = pro-inflammation protein cocktail of r*Aam*IGFBP-rP1, r*Aam*IGFBP-rP6L, r*Aam*IGFBP-rP6S. PI1, PI2, and PI3 = 0.1, 1 μg, and 10 μg of the PI cocktail. AI = anti-inflammation protein cocktail of rAAS27 and sAAS41. AI1, AII2, and AI3 = 0.1, 1 μg, and 10 μg of the AI cocktail. T1, T2, T3, T4 and T5 represents each experiment.

PI-rTSPs activate MΦ via TLR4 and cytokine signaling pathways

The observation that PI-rTSP stimulated MΦ to express high levels of co-stimulatory markers, pro-inflammation cytokines, and secretion of NO fit the phenotype of classically activated (or M1) MΦ (Martinez and Gordon 2014). Alternatively, induction of anti-inflammatory cytokines by AI-rTSPs suggest that these proteins might have induced alternatively activated (or M2) MΦ, which have anti-inflammatory phenotype (Roszer 2015). As M1 MΦ were activated via the TLR4 or cytokine receptor signaling pathways (Italiani and Boraschi 2014), expression of signaling markers for these two pathways was investigated (Fig.8). Data in Fig.8 demonstrates that the three PI-rTSPs may activate MΦ via the TLR4 and cytokine signaling pathways. Except for NFκB1 (Fig.8A), three, NFκB2, RelA, and RelB (Fig.8B, 8C, and 8D) of the four tested TLR4 signaling markers and two cytokine signaling, cFos and cJun (Fig.8E and 8F) were differentially up regulated in a dose responsive manner when MΦ were co-incubated with various dosages of the PI-rTSP cocktail. Interestingly, adding AI-rTSPs to these MΦ suppressed NFkB transcript levels by more than 50% (Fig.8A) and slightly for other markers. Additionally, AI-rTSP cocktail induced significant secretion of the anti-inflammation cytokine, TGFβ and IL10 to MΦ that were pre-activated with PI-rTSP. This was also confirmed when qRT-PCR analysis of markers for the anti-inflammation M2 MΦ was significantly induced (Fig.8).

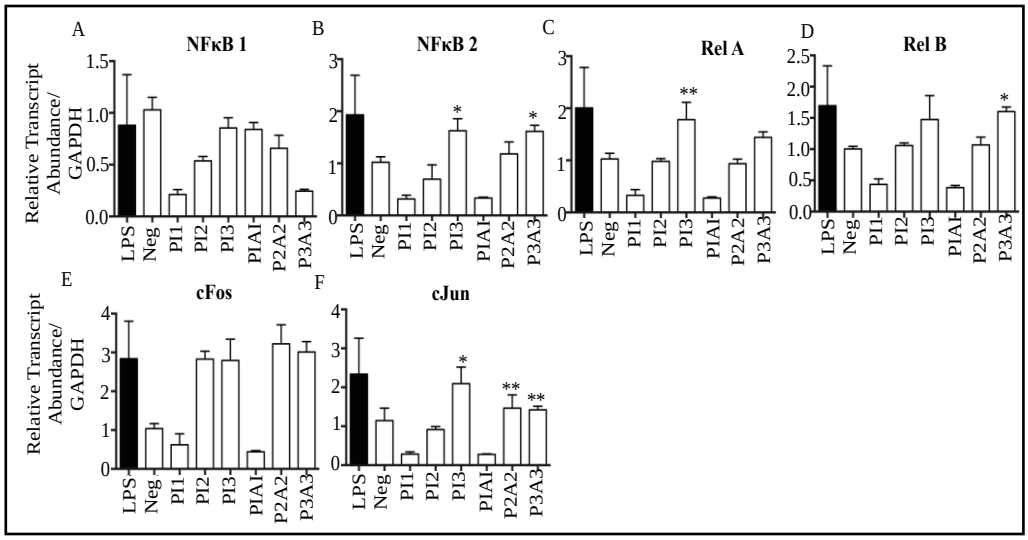


Figure 8. The pro-inflammation (PI)-rTSP (r*Aam*IGFBP-rP1, r*Aam*IGFBP-rP6S, and r*Aam*IGFBP-rPL6) cocktail induced expression of markers for pro-inflammatory macrophage activation. Macrophages were cultured in medium containing with various doses of the PI-rTSP cocktail only (PI) or the PI-rTSP and anti-inflammation (AI)-rTSP cocktail (PIAI). Cells were processed for qRT-PCR analysis using primers in Table 6. Transcript abundance of markers for pro-inflammatory macrophage activation, NFκB1 (Nuclear factor kappa light chain enhancer of activated B cells), NFκB2, RelA (Transcription factor p65 or Nuclear factor kappa B p65) , RelB, Activator protein molecules: cFos and cJun was determined by qRT-PCR as descibed. GAPDH was used as internal reference and Media only was used as calibrator. Data is presented as fold increase and/or decrease from the media as the mean ± SE of three biological replicates. (*) = $p \leq 0.05$, (**) = $p \leq 0.01$, (***) = $p \leq 0.001$, (****) = $p \leq 0.0001$, indicating statistically significant difference between media and treatments using one way ANOVA followed by post hoc Dunnett's multiple comparisons test. PI1, PI2, and PI3 = 0.1, 1.0, and 10 μg of PI-rTSPs. PI1AI1, PI2AI2, and PI3AI3 = 0.1, 1.0, and 10 μg each of the PI- and AI-rTSP cocktails combined.

Discussion

Feeding success and competence of hard ticks to transmit tick borne disease (TBD) agents depends on multiple tick saliva proteins (TSP) that are injected into the host to evade host defense to tick feeding. Thus, discovery and determining functional roles of TSPs in tick feeding regulation is highly sought after as it will likely reveal critical proteins that will be targeted for development of novel methods to prevent

transmission of TBD agents (Simo et al., 2017; Ribeiro et al., 2017; Karim and Ribeiro 2015; Garcia et al., 2014; Karim et al., 2011; Francischetti et al., 2008; Konnai et al., 2010; Mudenda et al., 2014; Diaz-Martin et al., 2013). To this end, several approaches including immuno-screening of cDNA expression libraries (Mulenga et al., 1999; Ribeiro et al., 2006; Schwarz et al., 2014; Schwarz et al., 2013; Kotysfakis et al., 2015), biopanning of phage display cDNA expression (Radulovic et al., 2014, Lewis et al., 2015), and LC-MS/MS sequencing of proteins in tick saliva and tick cement (Hollman et al., *in press;* Kim et al., 2016, Tirloni et al., 2014; 2015, Vora et al., 2017, Bullard et al., 2016) were used to identify proteins that ticks inject into animals during feeding. The goal of the second chapter of this dissertation was to gauge insight into functional role(s) of 15 selected TSPs in tick feeding regulation.

The majority of published studies to understand how TSPs regulate the tick's evasion of host defense have involved assessing effects of crude salivary gland extracts (SGE) or defined rTSPs on immune cells that are pre-activated with mitogens such as the lipopolysaccharide (LPS), concanavalin (ConA), pokeweed (PWM), and phytohaemagglutinin (PHA) (Wikel et al., 1996; Cavassani et al., 2005; Kovar et al., 2001; Inokuma et al., 1998) or are pathogen activated (Banajee et al., 2016; Hermance and Thangamani 2015). Most of these studies have reported immunosuppressive effects in tick saliva. For instance, saliva of *Amblyomma variegatum* impaired the mobilization of inflammatory monocyte derived cells (Vachiery et al., 2015), while *R. microplus* saliva suppressed LPS activated MΦ expression of co-stimulatory markers including CD86 and CD69, but not CD40 and CD80 (Brake et al., 2010). The same authors found

that *R. microplus* salivary gland extracts suppressed MΦ secretion of pro-inflammatory cytokines. In another study of *D. variabilis* crude SGE inhibited migration of MΦ into the tick feeding site (Poole et al., 2013). In a related study, SGE of *D. variabilis* inhibited LPS activated MΦ secretion of pro-inflammatory cytokines (Poole et al., 2013). In this dissertation a different approach was utilized, investigating the direct effects of 15 recombinant (r)TSPs on MΦ function. This approach was based on the facts that: (a) not every tick bite is infectious, and (b) with the exception of some viruses (Hermance and Thangamani 2015), transmission of major tick-borne disease (TBD) agents predominantly occurs after ticks have fed for more than 48 h (Richards et al., 2017). Additionally, the infiltration of immune cells into the tick feeding site that starts within 24 h of tick attachment (Krause et al., 2009), precedes transmission of major TBD pathogens such as *Babesia bovis* and *B. bigemina* (Mahoney and Mirre 1977; Hodgson 1992), *Erhlichia ruminantium* (Uilenberg et al., 1993), *Borrelia burgdorferi* (Burgdorfer 1984), and *Anaplasma phagocytophilum* (Ogden et al., 2003), which are predominatly transmitted after the tick has fed for more than two days. From this perspective, there is likely an initial period during which immune cells are exposed to TSPs in the absence of TBD agents.

The macrophage (MΦ) is an important immune cell that plays key roles in regulating both innate (Fujiwara and Kobayashi 2005; Medzhitov and Janeway 1997; Parihar et al., 2010) and adaptive immunity (Scholl et al., 2016; Chen et al., 2012). Additionally, there is evidence that MΦ are associated with the tick feeding site as revealed by immunohistochemical staining of the tick feeding site that showed that MΦ

and other immune cells infiltrate the tick feeding site (Krause et al., 2009). On this basis, the observed effects of rTSPs on the function of MΦ could sufficiently be informative on roles of TSPs in tick feeding regulation. The functional roles of MΦ fall within a spectrum that is bordered by classically activated pro-inflammatory M1 MΦ and the alternatively activated anti-inflammatory M2 MΦ (Martinez and Gordon 2014). M1 MΦ are characterized by expression of pro-inflammation markers such as co-stimulatory markers CD40, CD80, and CD86 and cytokines, and secretion of nitric oxide (NO). The other phenotype is the alternatively activated M2 MΦ that are characterized by high expression of anti-inflammation cytokines that include IL-10 and TGFβ (Carreno and Collins 2002; Fujiwara and Kobayashi 2005). Expression of co-stimulatory markers on M1 MΦ is critical in shaping the extent and nature of immune response (Khan et al., 2012), which transforms MΦ to dendritic like cells to produce pro-inflammatory mediators TNFα, IL-6 and IL1 (Duque and Descoteaux 2015). TNFα is a pro-inflammation pleotropic cytokine that is important in fibroblast growth, adaptive immune response, T lymphocyte response, vasodilation and lymphocyte, neutrophil and monocyte infiltration during inflammation (Paraneswaran and Patial 2010). IL-6 is a key B cell and Th17 differentiation factor (Fernando et al., 2014). The interaction of B7 ligands CD80 (B7.1) and CD86 (B7.2) with their receptors are not only act as activation markers, they provide the most potent costimulatory signal for T cell activation (Salomon and Bluestone, 2001). CD80 expression correlates with the development of Th1 cells and CD86 expression favors the generation of Th2 cells (Kuchroo et al., 1995). Thus, the observation that similar to LPS, five *A. americanum* TSPs including

r*Aam*IGFBP-rP1, r*Aam*IGFBP-rP6S, r*Aam*IGFBP-rP6L, rAAS8, and r*Aam*TCI, stimulated MΦ to express co-stimulatory molecules, CD40, CD80, and CD86 in fig. 2 and pro-inflammatory cytokines, TNFα and IL-6 in fig. 3 indicated that these five rTSPs were pro-inflammatory (PI). On the other hand, two rTSPs, rAAS27 and rAAS41, which did not affect resting MΦ, but reversed LPS or PI-rTSPs activated MΦ were classified as anti-inflammatory (AI) proteins (Fig. 5-7). As endotoxins can stimulate MΦ into expression of pro-inflammation markers (Sweet and Hume 1996), there was a chance that the observed data was due to endotoxin contamination. This was ruled out in that both mammalian cell expressed PI-rTSPs devoid of endotoxin contamination as well as insect cell expressed PI-rTSPs stimulated MΦ to express pro-inflammation co-stimulatory markers (CD40, CD80, and CD86) and cytokines, TNFα, IL-6, and IL-1, and secretion of NO.

Our lab previously showed that *Aam*IGFBP-rP1, *Aam*IGFBP-rP6S, and *Aam*IGFBP-rP6L are structurally (Mulenga et al., 2013) and functionally similar as insulin binding proteins (Radulovic et al., 2014). In a related study, *A. americanum* tick saliva proteome analysis revealed that *Aam*IGFBP-rP1, *Aam*IGFBP-rP6S, and *Aam*IGFBP-rP6L have a similar secretion pattern (Kim et al., *in preparation*) during tick feeding. Thus, it was interesting to note that secretion levels of pro-inflammation cytokines were enhanced in MΦ that were treated with *Aam*IGFBP-rP1, *Aam*IGFBP-rP6S, and *Aam*IGFBP-rP6L cocktail suggesting these three PI-rTSPs had a synergistic pro-inflammatory effect. It is important to note that whereas individual PI-rTSPs did not stimulate expression of anti-inflammation cytokines, expression of anti-inflammation

cytokines, IL-10 and TGFβ was induced at high dosage of the PI-rTSP cocktail mix. This observation could be explained by the fact that at high dosage, the PI-rTSP cocktail over activated MΦ, which in turn triggered expression of anti-inflammation cytokines. There is evidence that an intense inflammation response is curtailed by expression of anti-inflammation cytokines including IL-10 and TGFβ to prevent destruction of self tissue (Deng et al., 2012, Sica and Mantovani 2012). Its most likely that the amount of PI-rTSPs that are injected into the host during tick feeding will be lower than amounts used in this study. On this basis, the observed expression of anti-inflammation cytokines at the high PI-rTSP cocktail dose may not be a physiological event.

In addition to inducing immune cell influx into the site of injury, inflammation also promotes blood flow to the site of injury through vasodilation and increased leakage of blood vessels (Freire and Dyke 2013). This actions would also result in increased blood flow to the tick feeding site. Thus, it is possible that the five PI-rTSPs described in this dissertation could be facilitating blood meal feeding through enhanced blood flow to the feeding site. Likewise, TNFα which was secreted at high levels in MΦ that were activated with individual PI-rTSP or cocktail mix of *Aam*IGFBP-rP1, *Aam*IGFBP-rP6S, and *Aam*IGFBP-rP6L was shown to promote blood flow to the site of injury (Naredi et al., 1993; Farkas et al., 2006).

As the tick is expected to suppress the host's inflammatory response to successfully feed and transmit TBD agents (Wu et al., 2010; Glatz et al., 2017; Simo et al., 2017), data in this dissertation which showed that *A. americanum* PI-rTSPs promoted inflammation was counter-intuitive. However, data presented here is not unique to this

study as pro-inflammation activities have been previously documented in tick saliva. A functional tick histamine release factor (Mulenga et al., 2003) was suspected to promote inflammation through release of histamine, an agonist for acute inflammation (Benly 2015). Likewise, a serine protease 84 kDa inducer of immediate hypersensitivity in naïve rabbits was purified from *Haemaphysalis longicornis* (Mulenga et al., 2001). In a recent study, Powassan virus infected *I. scapularis* ticks induced expression of markers for inflammation during the first 2 h of feeding on mice (Hermance and Thangamani 2015).

We expected ticks to suppress host defense (Kazimirova and Štibrániová 2013; Wikel 2013). Thus, the observation that rAAS27 and rAAS41 reversed the expression of pro-inflammation markers by both LPS and PI-rTSPs stimulated MΦ was not surprising. Several studies have reported tick immune-suppressive effects (Kotysfakis et al., 2006; Oliveira et al., 2008; Guo et al., 2009; Langhansova et al., 2015; Wang et al., 2016; Chmelar et al., 2011). However, the observation was that both rAAS27 and rAAS41 did not act on resting MΦ, but they acted on pre-activated MΦ was interesting. Another notable observation was that co-incubating the rAAS27 and rAAS41 cocktail with MΦ that were pre-activated with either PI-rTSPs, but not LPS, enhanced secretion of TGFβ anti-inflammation cytokine. From the perspective of TBD transmission, the observation that rAAS27 and rAAS41 likely acted on pre-activated MΦ is interesting. TBD agents such as *Ehrlichia chaffeensis* and most tick-borne viruses (TBV) target and activate MΦ to express pro-inflammatory cytokines (Miura et al., 2011; Hermance and Thangamani 2014; Scholl et al., 2016). Studies have shown that activated MΦ can limit proliferation

of TBV (Tick borne virus), such as tick-borne encephalitis (Ahantarig et al., 2009). Could it be possible that AAS27 and AAS41 could act on tick borne disease (TBD) agents activated MΦ to control the killing of TBD agents, and as a consequence enhance transmission?

LPS, which was used as the positive control in this research activates MΦ toward the M1 phenotype via the toll-like receptor (TLR)4 (Lu et al., 2008). The observation that similar to LPS, treatment of MΦ with PI-rTSPs induced expression of markers for TLR4 signaling could suggest that tick induced inflammation may be mediated via the TLR4 pathway. It is also possible that PI-rTSPs could have have mediated MΦ activation via the cytokine signaling pathway, which intersects with TLR4 signaling pathway.

In conclusion, this second chapter shows that *A. americanum* ticks secretes two types of saliva proteins: (i) those that promote inflammation here referred to as PI-rTSPs, and (ii) those suppress inflammation, here called as anti-inflammatory, AI-rTSPs. Based on findings in this chapter, I propose a scenario of where both PI-rTSPs and AI-rTSPs are part of the tick's mechanism to evade host defense. Although I used RAW macrophages, which do not mimic primary cell function, I speculate that, the tick may utilize a strategy that is similar to the "bait and switch" mechanism on host macrophages, of where PI-rTSPs activate MΦ, and AI-rTSPs dampen those activated MΦ to where their effect in directing the host's response to tick feeding does not proceed as intended.

Overall, findings in this second chapter advances our understanding of how ticks

may evade host defense. The discovery of defined TSPs that interact with MΦ adds to the complexity of tick feeding physiology. As this second chapter was cell culture based, I proceeded to investigate if both AI- and PI-rTSP described in this second chapter were functional *in vivo* in the third chapter.

CHAPTER III

VALIDATE *IN-VIVO* FUNCTIONS OF *IN-VITRO* VALIDATED PRO/ANTI-INFLAMMATORY RECOMBINANT TICK SALIVA PROTEINS

Introduction

In chapter II of this dissertation, an *in vitro* cell culture based analysis investigated the effect of 15 recombinant tick saliva proteins (rTSPs) on murine RAW 264.7 macrophage (MΦ) expression of pro-inflammation co-stimulatory markers, CD40, CD80, and CD86, pro-and anti-inflammation cytokines, and secretion of nitric oxide. This analysis identified five rTSPs (*Aam*IGFBP-rP1, *Aam*IGFBP-rP6S, and *Aam*IGFBP-rP6L, AAS8, and *Aam*TCI) that were classified as pro-inflammation (PI) rTSPs as they stimulated MΦ to express pro-inflammation co-stimulatory markers and cytokines. Two other rTSPs, rAAS27 and rAAS46 were classified as anti-inflammatory (AI) as they reversed the expression of pro-inflammation markers in both LPS and PI-rTSP activated MΦ. In a separate study, our lab has determined that both PI- and AI-rTSPs that were characterized in the second chapter are injected into animals during tick feeding (data not shown). The goal of this second chapter was to determine if cell culture validated PI- and AI-rTSP were functional *in vivo* using the mouse hind paw edema assay (Winter et al., 1962).

Paw or footpad swelling was first used as convenient method for assessing inflammatory responses more than 50 years ago (Winter et al., 1962). This approach is now a routine method to demonstrate functionality of both agonists (Vajja et al., 2004, Morris 2003, Waisberg et al., 2014) and antagonists (Oyanagui et al., 1991; Hassimotti

et al., 2013) for inflammation. Our lab and our collaborators in Brazil, used paw edema to demonstrate that rAAS27 and AAS41 individually blocked formalin and compound 48/80 induced inflammation in rats (Kim et al., *in preparation,* Tirloni et al., *in preparation*). In this third chapter of the dissertation, the effects of rAAS27 and rAAS41 on PI-rTSP induced inflammation in an in-vivo foot pad swelling assay was investigated.

Materials and Methods

Paw edema assay

The paw edema assay was performed as previously described (Winter et al., 1962) using female retired breeder BALB/c mice (Envigo, Huntingdon, UK). Animals were housed and cared for under humane conditions according to the approved animal use protocol (#2015-0079) by Texas A&M University Institutional Animal Care and Use Committee (IACUC) under pathogen free conditions in temperature controlled rooms, receiving water and food ad libitum. Edema progression was studied by measuring the baseline thickness of the hind footpads using plythesmometer (Harvard Apparatus, Holliston, MA). Four experiments were conducted. Groups of three mice was used in all four experiments (Table 8). In the first experiment, combinatorial effect of PI-rTSPs were assessed. Approximately 40 µg each of endotoxin free mammalian cells expressed r*Aam*IGFBP-rP1, r*Aam*IGFBP-rP6L and r*Aam*IGFBP-rP6S were combined, and concentrated to 25 µg using Jumbosep centrifugal spin filter devices (Pall Life Sciences, Post Washington, NY). The first group of mice received 25 µg of r*Aam*IGFBP-rP1, r*Aam*IGFBP-rP6S and r*Aam*IGFBP-rP6L (PI group) cocktail. The second group of mice received a 25 µg cocktail AAS27 and AAS41 proteins cocktail (AI group), third group

received 25 µg of the AI-rTSP and PI-rTSP cocktail.

In the second experiment, the effects of individual rTSPs were assessed. Each group of mice received 40 µg of individual PI- and AI rTSPs. In the third experiment, less protein was used, cocktails of both PI-rTSP and AI-rTSP were made by mixing 10 µg of the each of the proteins. The first group of mice received a cocktail of 10 µg of each PI rTSPs, the second received combinations of the each of 10 µg of AI- and PI-rTSP cocktails, the third group received AI-rTSPs, PI combination with 10ug AAS41, the fourth group received 10 µg of rAAS27 or rAAS46, the fifth group received 10 µg of combination of AI-rTSPs and 2% carrageenan. In the fourth experiment, group of mice received each of 10 µg each of PI-rTSPs. Each treatment was injected in 25 µL volumes using 25 guage needles (Beckton Dickinson, Franklin Lakes, NJ) (Table 8).

For all experiments, algae derived inflammation agonist, carrageenan (2% w/v in 0.9% saline) was used as positive control and normal saline (0.9% NaCl only) as negative control in all experiments. Inflammation was measured as difference in paw thickness at 0 minute and the normal saline injected group. For the first and second experiment, the footpad swelling was measured at times 0, 20, 40, 60, 120, 240, 360, 720 and 1440 minutes post injection. For the third and fourth experiments, paw edema was measured at 0, 20, 40, 60, and 120 minutes post injection. After measurements, the mice were euthanized (3L/minute, CO_2) and snap frozen in liquid nitrogen. The paws were collected at the level of calcaneus bone for cytokine, chemokine, and myeloperoxidase assays (below).

Table 8 Treatment groups for paw edema assay in four experiments

Experiment (h)	**Treatments groups (n=3)**	**Dose injected**	**Diluent**
1- 24 h	Carageenan (CAR)	2%	Normal Saline
	Normal saline (S)	0.9%	Normal Saline
	PI-rTSPs	25µg	150mM 50mM Tris Nacl buffer (pH 7.4)
	PI-AI rTSPs	25µg PI-rTSPs + 25µg AI-rTSPs	Tris Nacl buffer
2 – 24 h	Carageenan	2%	Normal Saline
	S	0.9%	Normal Saline
	*Aam*IGFBP-rP1	25µg	150mM 50mM Tris Nacl buffer (pH 7.4)
	*Aam*IGFBP-rP6 L	25µg	150mM 50mM Tris Nacl buffer (pH 7.4)
	*Aam*IGFBP-rP6 S	25µg	150mM 50mM Tris Nacl buffer (pH 7.4)
3 – 2 h	Car	2%	150mM 50mM Tris Nacl buffer (pH 7.4)
	S	0.9%	150mM 50mM Tris Nacl buffer (pH 7.4)
	PI-rTSPs	10µg	150mM 50mM Tris Nacl buffer (pH 7.4)
	PI-AI rTSPs	10µg	150mM 50mM Tris Nacl buffer (pH 7.4)
	P+AAS27	10µg PI-rTSPs + 10µg AAS27	150mM 50mM Tris Nacl buffer (pH 7.4)
	P+AAS41	10µg PI-rTSPs + 10µg AAS41	150mM 50mM Tris Nacl buffer (pH 7.4)
	CA	2% Car + 10µg AI-rTSPs	150mM 50mM Tris Nacl buffer (pH 7.4)
	AAS27	10µg	150mM 50mM Tris Nacl buffer (pH 7.4)
	AAS41	10µg	150mM 50mM Tris Nacl buffer (pH 7.4)
4- 2 h	Car	2%	150mM 50mM Tris Nacl buffer (pH 7.4)
	S	0.9%	150mM 50mM Tris Nacl buffer (pH 7.4)
	*Aam*IGFBP-rP1	10µg	150mM 50mM Tris Nacl buffer (pH 7.4)
	*Aam*IGFBP-rP6 L	10µg	150mM 50mM Tris Nacl buffer (pH 7.4)
	*Aam*IGFBP-rP6 S	10µg	150mM 50mM Tris Nacl buffer (pH 7.4)

Quantitative RT-PCR analysis of pro-inflammation co-stimulatory markers, pro- and anti-inflammatory cytokines, and chemokines in edematous tissues

The paws from above were minced in 1 mL TRIzol solution using soft tissue scissors followed by sonication using the tissue dismembranator (Thermo Scientific, Wilmington, DE). Total RNA was isolated using TRIzol ® method (Thermo Scientific, Wilmington, DE). The organic phase during total RNA isolation contained the rest of the tissues and were discarded. Following total RNA isolation, mRNA isolation, cDNA synthesis was done as described (Thermo Scientific, Wilmington, DE). Template cDNA was synthesized from 25 ng mRNA using the Verso cDNA synthesis kit ®, following the manufacturer's protocol (Thermo Scientific, Wilmington, DE). Routinely, qRT-PCR was performed as described in chapter 2. All primer sets (forward and reverse) was 300 nM in concentration. The mRNA expression were determined by delta delta Ct (Livak and Schmittgen 2001) method. The GAPDH was used as internal control and normal saline injected group was used as calibrator. The primers used in the study are listed in Table 9.

Table 9 Quantitative RT-PCR primers for pro/anti inflammatory cytokine, chemokine and cell surface marker expression

Target	Primer sequence
TGFβ	For: 5′-TGGAGCAACATGTGGAACTC-3′ Rev: 5′-TGCCGTACAACTCCAGTGAC-3′
CXCL1	For: 5′-GACCATGGCTGGGATTCACC-3′ Rev: 5′-CCAAGGGAGCTTCAGGGTCA-3′
CCL2	For: 5′-CCGGCTGGAGCATCCACGTGT-3′ Rev: 5′-TGGGGTCAGCACAGACCTCTCTCT-3′
CCL5	For: 5′-ATATGGCTCGGACACCACTC-3′ Rev: 5′-TCCTTCGAGTGACAAACACG-3′
CCL11	For: 5′-CCAGGCTCCATCCCAACTT-3′ Rev: 5′-TGGTGATTCTTTTGTAGCTCTTCAGT-3′
TNF-α	For: 5′-ATGAGCACAGAAAGCATGA-3′ Rev: 5′-GAATGAGAAGAGGCTGAGA-3′
IL-6	For: 5′-CTCTGGGAAATCGTGGAAAT-3′ Rev: 5′-CCAGTTTGGTAGCATCCATC-3′
IL-1	For:5′-CAACCAACAAGTGATATTCTCCATG-3′ Rev: 5′-′GATCCACACTCTCCAGCTGCA-3′
IL-12	For: 5′-GGGAAGACAATAACTGCACC-3′ ′ Rev: 5′-′GCTGGTCCTTTGTTTGAAAGA-3′
IL-10	For: 5′-ATGGTGAAGACGGCCAGA-3′ Rev: 5′-CAGGTCTTCAATGTGCTGGTT-3′
CD40	For: 5′-GCTATGGGGCTGCTTGTTGA-3′ Rev: 5′-ATGGGTGGCATTGGGTCTTC-3′
CD80	For: 5′-CTGGGAAAAACCCCCAGAAG-3′ Rev: 5′-TGACAACGATGACGACGACTG-3′
CD86	For: 5′-CATGGGCTTGGCAATCCTTA-3′ Rev: 5′-AAATGGGCACGGCAGATATG-3′
GAPDH	For: 5′′ATGTCGTGGAGTCTACTGGT3′ Rev: 5′-GAGTTGTCATATTTCTCGT-3′

Myeloperoxidase assay

Leukocyte migration into edematous tissue was done by myeloperoxidase assay using the myeloperoxidase (MPO) colorimetric assay kit. (Sigma-Aldrich, St. Louis, MO). Myeloperoxidase (MPO) is a heme containing enzyme that catalyzes the hydrogen peroxidase mediated oxidation of halide ions to hypohalous acid (Agner 1941; Harrison

and Schultz 1976). It is a lysosomal protein and is highly expressed in neutrophils and is used to detect the level of neutrophils in the tissue (Clark et al., 1987). The half hind paw was cut into small pieces and homogenized in the 100 μL MPO buffer as per recommended by manufacturer's protocol and centrifuged at high speed. Aliquots of each supernatant were transferred to 96 well plate and MPO substrate and TNB (5-thio-2 nitro benzoic acid) was added. MPO catalyzes the formation of hypochlorous acid, which reacts with taurine to form taurine chloramine, which reacts with chromophore TNB, resulting in the formation of colorless product DTMB. The absorbance of color change was detected at 412 nm. In parallel, dilutions of TNB standards were used for myeloperoxidase standard curve (OD as a function of units of enzyme activity). The MPO activity was measured using the formula: MPO Activity = B x Sample Dilution Factor/(Reaction Time) x V, B = Amount (nmole) of TNB consumed, Reaction Time = (in minutes, at point stop mix was added), V = sample volume (mL) added to well.

Statistical Analysis

One-way ANOVA followed by Dunnett's post hoc test was used to determine the statistical differences between the controls and treatments using the Prism 6 and 7 software (GraphPad Software, La Jolla, CA, USA) (Chmelar et al., 2011, Chimedza et al., 2017, Sakat et al., 2013, Oz et al., 2017). The statistical significance of mRNA transcript abundance was determined for each replicate using the formula $2^{-\Delta\Delta CT}$ as described by Livak and Schmittgen, 2001.

Results

Cell culture validated pro-inflammation and anti-inflammation rTSPs are functional in vivo

Four experiments summarized in Figs.9A-9D show that the mouse hind paw edema assay was successfully used to demonstrate that cell culture validated pro-inflammatory (PI, r*Aam*IGFBP-rP1, r*Aam*IGFBP-rP6L, and r*Aam*IGFBP-rP6S) and anti-inflammatory (AI; rAAS27 and rAAS41) rTSPs were functional *in vivo*. As indicated in Fig.9, mice were injected with a high dose of the cocktail or individual PI-rTSPs in the first and second experiments (Figs.9A and 9B), while in the third and fourth experiments, mice were injected with the low rTSP dose. In all experiments, the algae derived inflammation agonist, carrageenan (2%) was used for positive control, and normal saline (9 g/L NaCl) for negative control. As shown in Fig.1A injecting mice hind paws (n=4) with 25 μg of the high dose cocktail of the three PI-rTSPs (cocktail made by combining ~40 μg of each the three PI-rTSPs) induced edema as determined by paw swelling measured using Plythesmometer. The high dose PI-rTSP cocktail mix significantly induced progressive edema through 24 h that was comparable to that induced by 2% carrageenan (Fig.9A). Four peak edema points were observed at 20 min (0.0415 mL ± 0.003), which was equivalent to the carrageenan (2% in normal saline) positive control, at 240 min (0.030 ± 0.008 mL) and 480 min (0.025 ± 0.003 mL) and at 1440 min (0.02 ± 0.003 mL). When co-injected with the AI-rTSP cocktail, PI-rTSP induced edema was reduced by 32, 75, 66, and 100% at 120, 240, 480, and 1440 mins respectively (Fig. 9A). In the second experiment (Fig. 9B), mice (n=3) were injected

with high dose, 25 μg of the individual PI-rTSPs. As demonstrated in Fig.1B, all three PI-rTSPs were functional pro-inflammation proteins as they induced edema. As shown in Fig.9B, r*Aam*IGFBP-rP6L induced edema that peaked at 480 min (0.013 ± 0.008 mL) and 1440 min (0.008 ± 0.003 mL), while r*Aam*IGFBP-rP6S induced edema peaked at 20 min (0.015 ± 0.005 mL), 40 min (0.015 ± 0.005 mL), 240 min (0.008 ± 0.003 mL), and 1440 min (0.010 ± 0.005 mL). In the third (Fig.9C) and fourth (Fig.9D) experiments, mice were injected with the low dose 10 μg of the cocktail (made by combining 10 μg of each of the PI-rTSPs) or 10 μg individual PI-rTSPs. For the third and fourth experiments edema was monitored for 120 min (Fig. 9C and 9D). As shown in Fig.9C, peak swelling in mice feet that were injected with the low dose cocktail was observed at 20 min (0.033 ± 0.000 mL) and 60 min (0.042 ± 0.002 mL). The administration of AI and PI cocktail inhibited edema formation by 90, 72,76 and 66% at 20, 40, 60 and 120 min (Fig. 9C). Fig.1D demonstrates that injecting mice with low dosages of individual PI-rTSP induced edema. As shown in Fig.9D, paw swelling (0.009 ± 0.003 mL) was apparently above negative control at the 120 min time point in mice that were injected with r*Aam*IGFP-rP1. For r*Aam*IGFBP-rP6S and r*Aam*IGFBP-rP6L, paw swellings of 0 to (0.009 ± 0.009 mL) , and (0.007 ± 0.003 mL) to (0.037 ± 0.010 mL) were observed from 20 to a 120 min respectively (Fig. 9D) confirming that even at the low dose, these two proteins were pro-inflammatory.

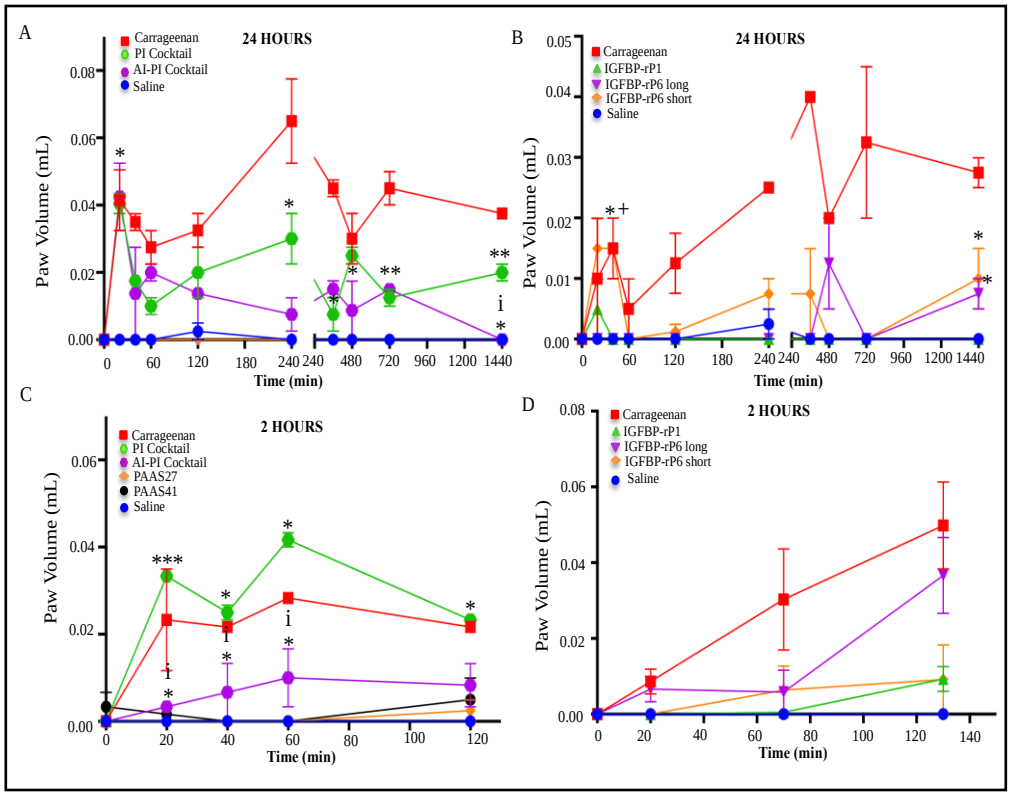


Figure 9. Cell culture characterized pro-inflammation (PI) and anti-inflammation (AI)-recombinant tick saliva proteins (TSPs) are functional *in vivo*. Cocktail or individual pro-inflammatory (PI), r*Aam*IGFBP-rP1, r*Aam*IGFBP-rP6L, and r*Aam*IGFBP-rP6S or anti-inflammatory (AI), rAAS27 and rAAS41 rTSPs were injected into mice hind paws as described in materials and methods. Mice in Figs.9A and 9B received the high dose of cocktail and individual protein respectively, while mice in Figs.9C and 9D received the low dose. Paw thickness as an index for the level of inflammation was done at 20, 40, 60, 120, 240, 480, 720 and 1440 min (Figs. 9A, and 9B), and at 20, 40, 60 and 120 min (Fig. 9C and D). Carrageenan and sodium chloride were used for positive and negative control. Normal saline values were subtracted from all values plotted in Fig.1. Data is represented as mean of three biological replicates ± SEM. Filled squares, red (■) = carrageenan injected, filled circles, blue (●) = normal saline, filled hexagon, green (⬣) = high dose PI-rTSP cocktail, purple (⬣) = high dose PI-rTSP cocktail co-injected with high dose AI-rTSP cocktail, Filled triangle (▲) = r*Aam*IGBP-rP1, Upside down , purple▼) = r*Aam*IGBP-rP6L, filled diamond, orange (◆) = r*Aam*IGBP-rP6S (B and D), PI-rTSP cocktail and rAAS27 (C) , filled circles, black (●) = PI-rTSP cocktail and rAAS41. r*Aam*IGFBP-rP = *A. americanum* insulin-like growth factor binding protein-related protein, rAAS27 and rAAS41 = *A. americanum* tick saliva serpin 27 and 41. (*) represents p-value <0.05 indicating statistically significant difference between normal saline injected group and treatments groups using ANOVA Dunnett's multiple comparisons test. (* i) represents p-value <0.05 indicating statistically significant difference between PI-rTSPs injected group and PI-AI rTSPs injected group using ANOVA Dunnett's multiple comparisons test. No asterisks means non-significance.

PI-rTSP induced edema is accompanied by high expression of pro-inflammation, co-stimulatory markers, cytokines, and chemokines

One half of the paws in experiments summarized in Figs. 9A-9D were subjected to quantitative RT-PCR expression analysis of pro-inflammation co-stimulatory markers (Fig.10), pro- and anti-inflammation cytokines (Fig.11), and chemokines (Fig.12). As shown in Fig. 10A the CD40 expression was significantly high in 2 h harvested paws that were injected with low dose r*Aam*IGFBP-rP1 and r*Aam*IGFBP-rP6S, but not r*Aam*IGFBP-rP6S. In contrast CD40 expression was low in 24 h paws that were injected with high dose individual PI-rTSPs, except for r*Aam*IGFBP-rP1 (Fig. 10C). Unlike in 2 h harvested paws in which both r*Aam*IGFBP-rP1 and r*Aam*IGFBP-rP6S induced CD40 expression significantly above background (Fig. 10A), expression was significantly lower in 24 h harvested paws that were injected with high dose individual PI-rTSPs (Fig. 10B), except for those that were co-injected with PI-rTSPs, rAAS27 and rAAS41 and low dose PI.

Interestingly, CD40 was induced significantly above background in 24 h paws that were injected the high dose PI-rTSP cocktail, but not those that were co-injected with the AI- and PI-rTSP cocktail (Fig. 10D). For CD80, transcription was significantly above background in 2 h harvested paws that were injected with low dose r*Aam*IGFBP-rP1 and r*Aam*IGFBP-rP6S, but not in 2 h harvested paws that were injected with low dose cocktail PI-rTSP (Fig. 10E and 10F). In 24 h paws, CD80 transcription was significantly induced in 24 h harvested paws that were injected with the r*Aam*IGFBP-rP1 high dose as well as the high dose PI-rTSP cocktail (Fig. 10G and 10H). For CD86, transcription was induced significantly above background in 2 h harvested paws injected with the three individual PI-rTSPs, but those injected with the low dose PI-rTSP cocktail (Fig. 10I and 10J). Likewise, significant expression of CD86 transcript was observed in 24 h harvested paws that were injected with the high dose of r*Aam*IGFBP-rP1 or PI-rTSP (Fig. 10K and 10L). As shown in Figs. 10D, 10H and 10L, co-injecting the high dose PI-rTSP with the AI-rTSP cocktail significantly suppressed transcription of CD40, CD80 and CD86.

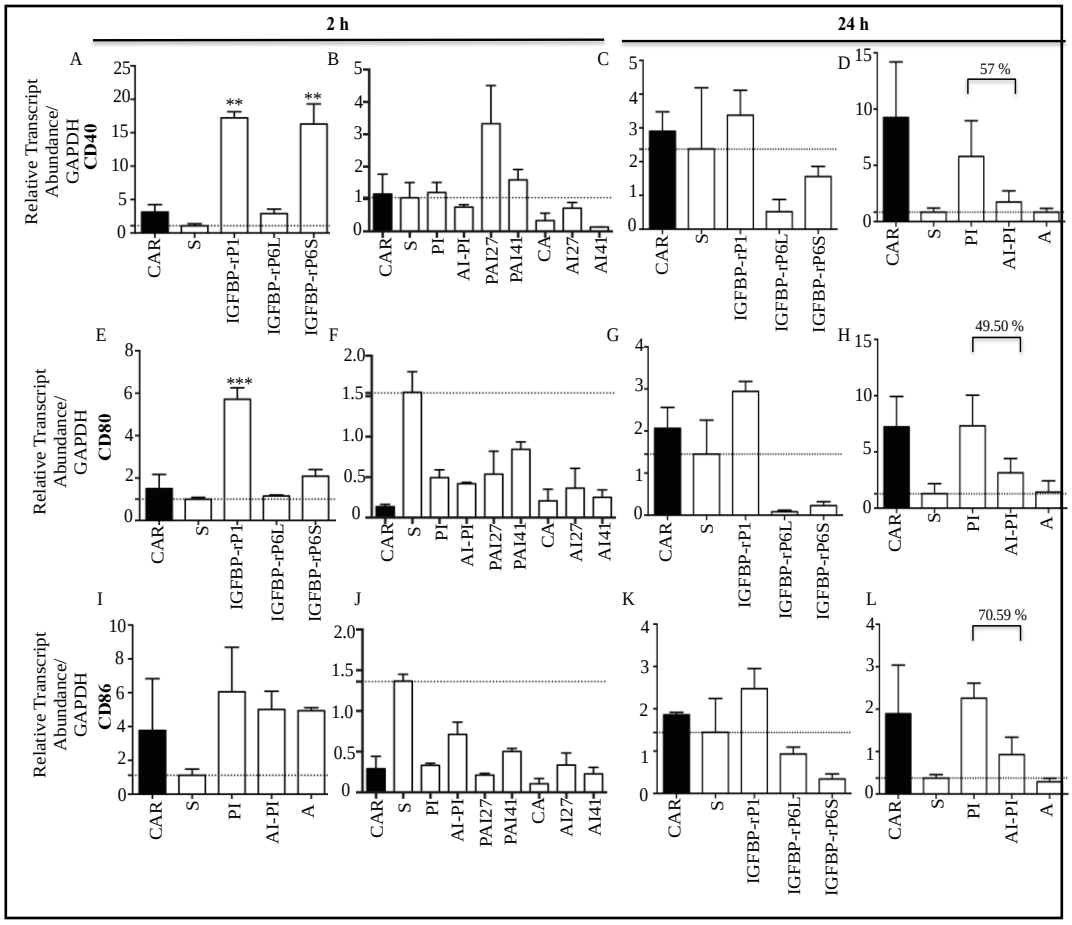


Figure 10. Pro-inflammation rTSP (individual or cocktail) induced edema associated with high expression of pro-inflammation co-stimulatory markers. Total RNA that extracted from hind paws that were injected with low dose (2 h paws) and high dose (24 h paws) PI-rTSP and AI-rTSP was subjected to qRT-PCR analysis to determine the expression of pro-inflammatory co-stimulatory markers, CD40, CD80 and CD86. Utilizing glyceraldehyde-3-phosphate (GAPDH) gene as the normalizer, relative transcript abundance was determined using comparative Ct (ΔΔ Ct) method as described. Relative fold change was calculated in comparison with normal saline values. Data is reported as mean of three biological replicates ± standard error of mean (SEM) CAR = Carageenan (positive control), S = Normal saline, r*Aam*IGFBP-rP = *A. americanum* insulin-like growth factor binding protein-related protein, A127, and AI41 = *A. americanum* tick saliva serpin 27 and 41. PI = pro-inflammation rTSP cocktail, AI = anti-inflammation rTSP, PI-AI = PI and AI rTSP cocktail mix, PA127 = PI rTSPs and rAAS27 cocktail, PAI141 = PI rTSPs and rAAS41 cocktail mix, % = percentage sign shows the percent suppression. (*) represents p-value <0.05 indicating statistically significant difference normal saline injected group and treatments groups using one way ANOVA. No asterisks means non-significance.

Figs.11 and 12 demonstrate that PI-rTSP induced edema (Fig. 9) was accompanied by differential up regulation of pro-inflammatory cytokines and chemokines. As shown in Fig.11, the TNF-α transcript was significantly up regulated in 2 h harvested paws that were injected with low dose individual PI-rTSPs (Fig. 11A) and the cocktail PI-rTSP (Fig. 11B). As shown in Fig. 11C, the TNF-α transcript was not induced above background in 24 h harvested paws that were injected with the high dose of the individual PI-rTSPs, while significant expression was observed in those that were injected with the high dose of the PI-rTSP cocktail. Likewise, the IL-1 transcript was significantly up regulated in 2 h paws that were injected with the low dose of individual (Fig. 11E) or cocktail (Fig. 11F) of PI-rTSPs. Similarly, the IL-1 transcript was significantly induced in 24 h harvested paws that were injected with individual (Fig. 11G) or cocktail (Fig. 11H) PI-rTSPs, albeit at low levels when compared to 2 h harvested paws (Figs. 11E and Fig. 11F). In the case of IL-6, significant expression above negative control was observed in 2 h paws that were injected with individual PI-rTSPs, but not in 2 h harvested paws that were injected with the low dose cocktail (Fig. 11J). It is interesting to note that the profile of TGFβ (Fig. 11M-P), an anti-inflammation cytokine, although in low amounts, was comparable to pro-inflammation cytokines: TNFα and IL-1 in 2 h harvested paws that were injected with low dose individual proteins (Fig. 11M). It is also notable that co-injecting PI and AI-rTSP in 24 h harvested paws (Fig. 11P) did not affect TGFβ transcription

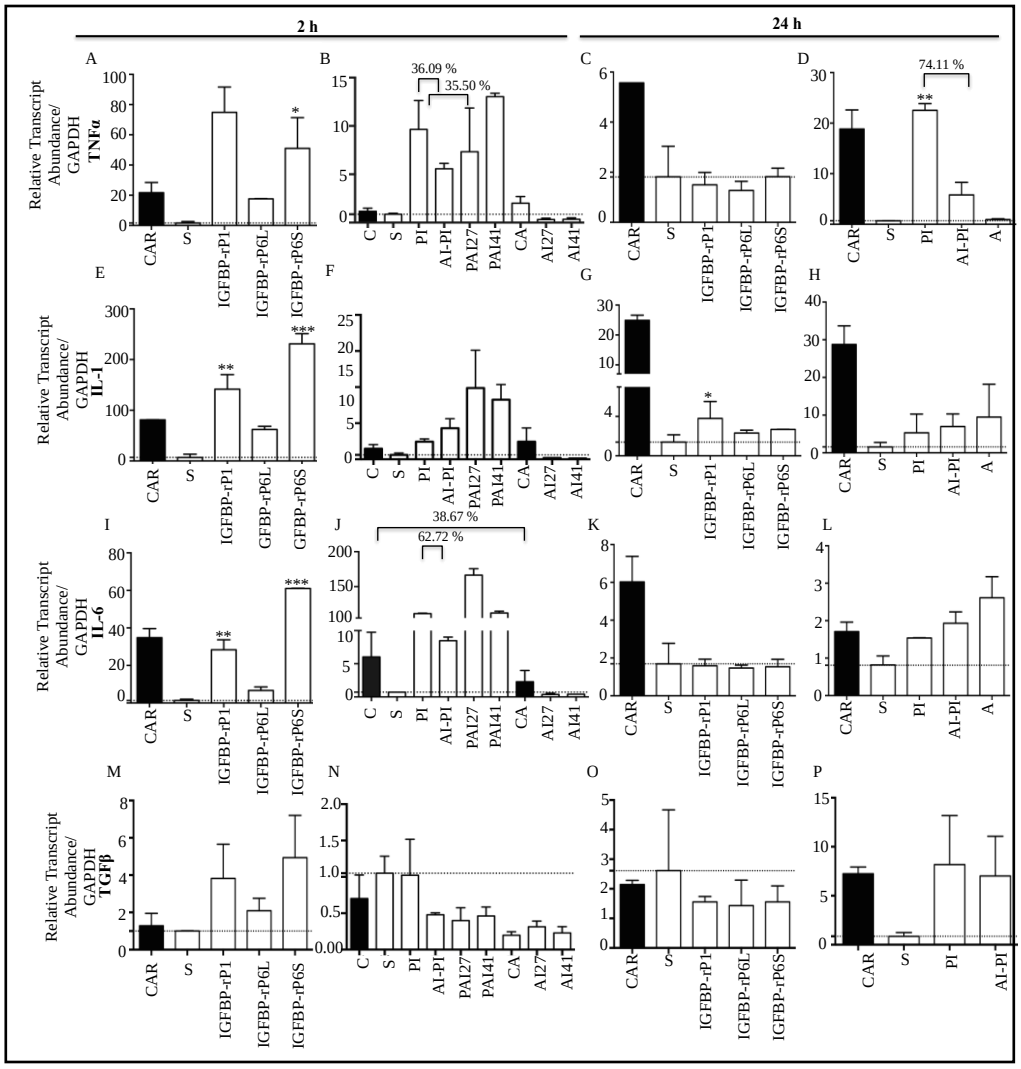


Figure 11. Pro-inflammation cytokines are significantly expressed in cocktail or individual pro-inflammation (PI)-rTSP induced edema. Total RNA that extracted from hind paws that were injected with low dose (2 h paws) and high dose (24 h paws) PI-rTSP and AI-rTSP was subjected to qRT-PCR analysis of pro-inflammatory cytokine, TNFα, IL1, IL6 and anti-inflammatory cytokine TGFβ. Normalized against glyceraldehyde -3-phosphate (GAPDH) for internal control, relative abundance of transcripts was determined using comparative Ct (ΔΔ Ct) method as described. Data is reported as Mean (M) of three biological replicates ± SEM. CAR = Carageenan (positive control), S = Normal saline, r*Aam*IGFBP-rP = *A. americanum* insulin-like growth factor binding protein-related protein, A127, and AI41 = *A. americanum* tick saliva serpin 27 and 41. PI = pro-inflammation rTSP cocktail, AI = anti-inflammation rTSP, PI-AI = PI and AI rTSP cocktail mix, PA127 = PI rTSPs and rAAS27 cocktail, PAI141 = PI rTSPs and rAAS41 cocktail mix, % = percentage sign shows the percent suppression. (*) represents p-value <0.05 indicating statistically significant difference between normal saline injected group and treatments groups using one way ANOVA. No asterisks means non-significance.

The effects of co-injecting PI- and AI-rTSPs on cytokine expression were mixed. In Fig.11B, TNF-α transcript abundance was respectively reduced by 36.09 and 35.50 % in low dose PI-rTSP cocktail 2 h harvested paws that were co-injected with the AI-rTSP cocktail and rAAS27. Similarly, co-injecting the AI-rTSP with the high dose PI-rTSP cocktail suppressed TNF-α transcript levels by 74.11 at 24 h% (Fig.11D). For IL-1, co-injecting AI-rTSPs, individually or as a cocktail had no effect, and appeared to slightly enhance IL-1 transcription in 2 and 24 h paws (Fig. 11F, H). Likewise, for IL-6, co-injecting AI-rTSP with PI-rTSPs suppress transcription level by 62.72% (Fig. 11J). However, co-injecting with rAAS27 in low dose PI-rTSP cocktail 2 h harvested paws (Fig. 11J) or with AI-rTSP cocktail in 24 h high dose PI-rTSP harvested paws (Fig. 11L) enhanced transcription.

We also assessed the effect of AI-rTSPs on carrageenan injected animals. Co-injection of AI-rTSPs with carrageenan did not change the transcripts of TNFα, IL-1 and TGFβ, However, the IL-6 was lowered to 38.67% respectively (Fig. 11J)

Fig. 12 summarizes the involvement of chemokines PI-rTSP induced inflammation. It was interesting to note that all chemokines investigated here were highly transcribed in 2 h paws that were injected with individual PI-rTSPs or the cocktail (Fig. 12A, 12B, 12E, 12F, 12I, 12J, 12M, 12Q and 12R) with the exception of CCL5 for which transcription could not be confirmed in 2 h paws that were injected with the low dose PI-rTSP cocktail.

It is notable that in both cocktail or individual PI-rTSP injected animals, the four chemokines, CXCL1, CCL2, CCL3 and CCL11 were expressed at much higher levels at 2 h than at 24 h (Fig. 12A, 12B, 12E, 12F, 12I, 12J, 12M, 12P, and 12Q). Whereas co-injecting cocktail or individual AI-rTSPs did not suppress cytokine expression of IL-1 (Fig.11 E, L), CXCL1, CCL2, CCL3 and CCL11 transcripts were significantly suppressed by up to 97.54, 99.51, 91.98. 95.43 % in 2 h harvested paws that were co-injected with cocktail or individual AI-rTSPs (Fig. 12B, 12F, 12J and 12R). Likewise, chemokine transcripts CXCL1, CCL2, CCL3 and CCL11 were also suppressed to 52.84, 47.52, 64.50 and 72.35 %in 24 h harvested paws.

In contrast expression of the CXCL5 chemokine was enhanced in animals that were co-injected with AI-rTSPs (Fig. 12P). While the effect of AI-rTSPs co-injected with PI-rTSPs was much higher, AI-rTSPs lowered CXCL1 expression in carrageenan induced inflammation (Fig. 12B)

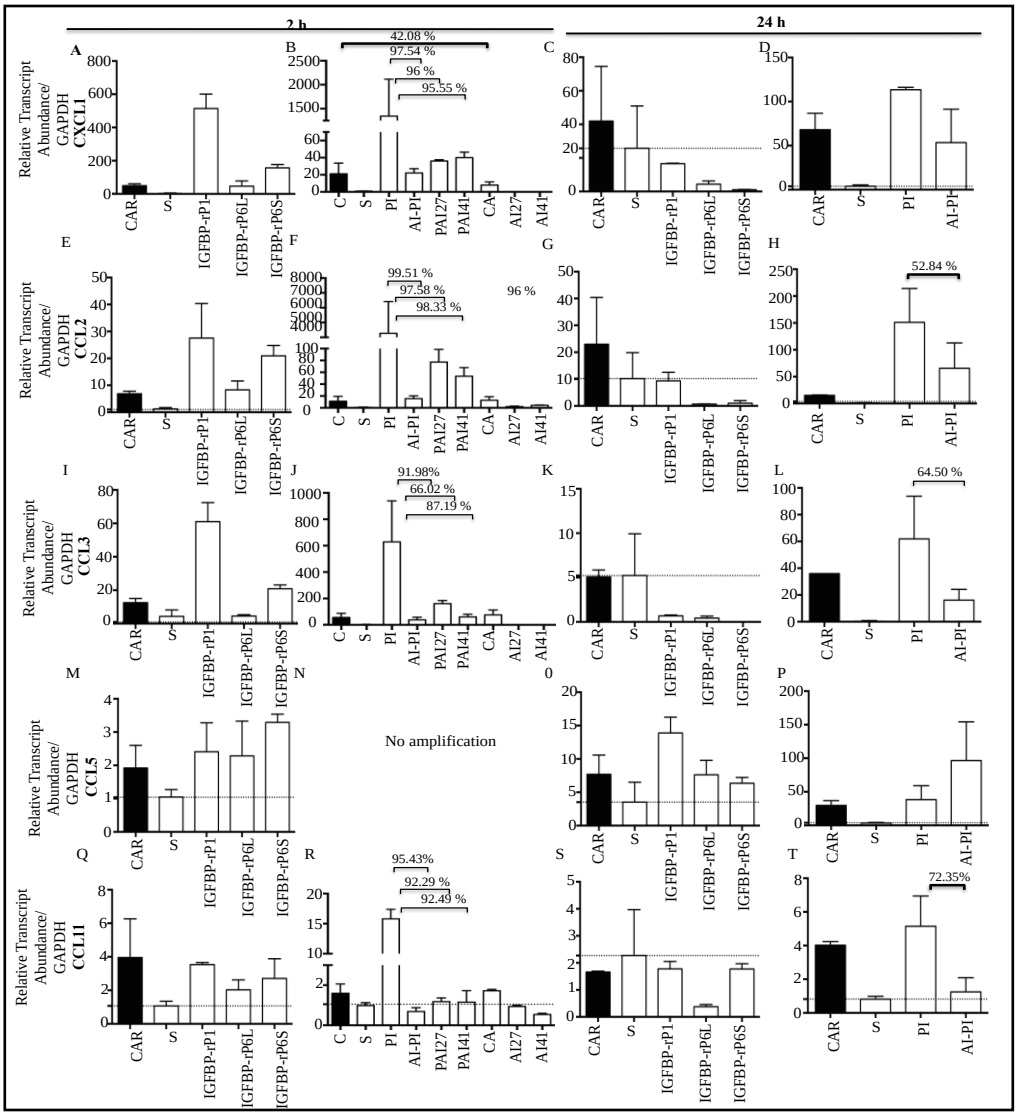


Figure 12. Chemokine transcripts are highly expressed at 2 h and 24 h in pro-inflammation rTSP induced edema. Total RNA that extracted from hind paws that were injected with low dose (2 h paws) and high dose (24 h paws) PI-rTSP and AI-rTSP was subjected to qRT-PCR expression analysis of chemokines, CXCL1, CCL2, CCL3, CCL5 and CCL11. Normalized against glyceraldehyde -3-phosphate (GAPDH) for internal control, relative abundance of transcripts was determined using comparative Ct (ΔΔ Ct) method as described. Data is reported as Mean of three biological replicates (M± SEM) CAR = Carageenan (positive control), S = Normal saline, r*Aam*IGFBP-rP = *A. americanum* insulin-like growth factor binding protein-related protein, A127, and AI41 = *A. americanum* tick saliva serpin 27 and 41. PI = pro-inflammation rTSP cocktail, AI = anti-inflammation rTSP, PI-AI = PI and AI rTSP cocktail mix, PA127 = PI rTSPs and rAAS27 cocktail, PAI141 = PI rTSPs and rAAS41 cocktail mix, % = percentage sign shows the percent suppression. (*) represents p-value <0.05 indicating statistically significant difference between normal saline injected group and treatments groups using one way ANOVA. No asterisks means non significance.

Discussion

The long-term research in the lab is to understand functions of tick saliva proteins at the tick feeding site. Thus, an important goal of this third chapter was to validate if *in vitro* validated pro-inflammation (PI) and anti-inflammation (AI) rTSPs were functional *in vivo.* Data presented demonstrated that at least three of five PI-rTSPs and the two AI-rTSPs that were identified in the second chapter were functional *in vivo.* It was interesting to note that similar to an algae derived inflammation agonist, carrageenan, the injection of r*Aam*IGFBP-rP1, *Aam*IGFBP-rP6S, and *Aam*IGFBP-rP6L individually or as a cocktail induced edema in mice hind paws, which was suppressed when mice were co-injected with the rAAS27 and rAAS41 cocktail. Our lab has recently demonstrated that rAAS27 (Tirloni et al., *in preparation*) and rAAS41 (Kim et al., *in preparation*) suppressed formalin and compound 48/80 induced edema in rats. These studies suggested rAAS27 and rAAS41 may block inflammation by respectively inhibiting pro-inflammation proteases, trypsin and chymase induced by these compounds, which is abundant in the skin. Whether or not these two proteases were associated with PI-rTSP induced edema was not investigated, however it would be interesting to confirm in future work.

Several studies have demonstrated that the most potent costimulatory signaling pathways in immunity and inflammation include CD28/B7 (CD80 and CD86) and CD40/CD40L (CD154) systems (Somaza and Lanier 1995; Brodskyn et al., 2001; Slawek et al., 2013). Expression of co-stimulatory molecules on the macrophages and other APCs is crucial for determining the nature and extent of the immune response

(Guermonprez et al., 2002; Santin et al., 1999). Furthermore, they play a major role in activation of T cells leading to proliferation, cytokine production and development of effector function (Somaza and Lanier 1995). We investigated the expression of co-stimulatory markers on macrophages by conducting systematic examination of costimulatory marker expression relating to cytokine and chemokine expression. Our results show that CD40, CD80 and CD86 was induced by individual PI-rTSPs at 2 h, albeit at low levels, at 24 h. This analysis shows that the expression of all three markers are in parallel, which shows that these markers express concurrently at the surface of antigen presenting cells. While the costimulatory markers are also expressed by various APCs (Kern et al., 2001; Zhan et al., 2003; Hellman et al., 2007), we limited our investigation to macrophage specific responses relating to effects observed *in vitro*.

Next, we were interested in looking at cytokine and chemokine responses following activation confirmed by cell surface marker expression. Our analysis showed that rTSPs induced high levels of TNF-α, IL-1, IL-6 at 2 h and low levels at 24 h. CD40, CD80, CD86 signal via NfKb to induce numerous cytokines like TNF-α, IL-1, IL-6 (Medzhitov 2001). Moreover, macrophages, when activated secrete prodigious amounts of pro-inflammatory chemokines to attract immune cells for pro-inflammatory activity (Fujiwara and Kobayashi 2005). We investigated the expression of few chemokine CXCL1, CCL2, CCL3, CCL5 and CCL11. CXCL1 is expressed by macrophages, neutrophils and epithelial cells and have neutrophil chemoattractant properties (Iida et al., 1990). CCL2 or monocyte chemoattractant protein 1 recruits monocytes, memory T cells and dendritic cells (Carr et al., 1994). CCL3, called

macrophage inflammatory protein 1-alpha is involved in recruitment and activation of poly-morphonuclear leukocytes (Wolpe et al., 1988). CCL5 is classified as chemotactic cytokine or chemokine for T cells, eosinophils and basophils (Donlon et al., 1990) and CCL11 or eotaxin is responsible for migration of eosinophil at the inflamed site (Kitaura et al., 1996). Chemokine expression acts within minutes, few hours and last up to 24 h (Ahn et al., 2007; Eberlein et al., 2010). Therefore, we observed the highest chemokine CXCL1 and CCL2 transcript at 2 h. The expression of chemokines CCL3, CCL5 and CCL11 was induced by individual and combination PI-rTSPs but sustained till 24 h. Therefore, our data confirmed the edema formation as a result of various cellular migration at the immune site. Although we did a systematic examination of different chemokines responsible for different cell migration, it would be of interest to examine the role of specific cell type at the injection site.

In addition to inflammatory activities, macrophages have the potential to contribute to remission of inflammatory episodes by inhibiting costimulatory markers and pro-inflammatory responses (Fujiwara and Kobayashi 2005). Either external signal by pathogens for longer survival (Weng et al., 2007) or the inhibitory mechanisms of the immune system shut the inflammatory signals from cells at inflamed site (Fujiwara and Kobayashi 2005). TGFβ produced by many cell types, in synergy with IL-10 play a crucial role in resolution of the response (Gong et al., 2012). Our results shows that TGFβ is induced at low levels for induction of anti-inflammatory effect. Both individual and combination PI-rTSPs induced TGFβ expression at low levels. Since TGFβ is a late phase expressing cytokine (Sadeghi et al., 2011), the effect was more at 24 h than 2 h,

indicating the effect of rTSP specific response and suppression of pro-inflammatory cytokine transcription at 24 h. On the other hand, AI-rTSPs also induce TGFβ but not more than PI-rTSPs. Interestingly, AI rTSPs had suppressive effect on TNF-α, IL-1, CXCL1, CCL2, CCL3 and CCL11 when co-injected with PI-rTSPs, showing an inhibitory response. Percent suppression was higher at 2 h compared to 24 h because of the highest pro-inflammatory response at 2 h. It was interesting to note that CCL5 expression was higher at groups injected with AI-PI rTSP at 24 h indicating possible synergistic effect. This could be possible for migration of T cells (Murooka et al., 2008). Therefore, the AI-rTSPs suppress the pro-inflammatory activities induced by PI rTSPs and the anti-inflammatory effect of AI-rTSPs may have been through different pathway than TGFβ signaling and remains to be investigated.

We were also interested in understanding the effects of AI-rTSPs on carrageenan induced edema. While there was no suppressive effect on co-stimulatory molecules, cytokines TNF-α, IL-1, TGFβ, chemokine CCL2, CCL3, CCL5 and CCL1, suppressive effects on IL-6, CXCL1 was observed indicating possible inference of AI-rTSPs on the carrageenan induced pathway. The effect of carrageenan inflammation is also shown to be suppressed by various molecules (Amdekar et al., 2012; Ahmad et al., 2016). Therefore, one possible effect is AI rTSPs affect carrageenan induced pathway by downregulating certain cytokines.

Previous studies demonstrated the influence of tick bites, tick saliva and its components on the innate and adaptive immune response (Hajnicka et al., 2005; Leboulle et al., 2002; Liu et al., 2014). However, these studies used *in vitro* and *in vivo*

assay using tick saliva or salivary gland extracts and may therefore not reflect the events induced by saliva molecules that might be very important in tick feeding. Only few studies of immune reaction to tick bites have been performed in mouse skin *in vivo*. For example, molecular investigations of early innate immune response in mice were found by upregulation of chemoattractants for monocytes, neutrophils and dendritic cells CXCL1, CXCL5, CCL2 and CXCL4 (Heinze et al., 2012). Consistent with these results, in BALB/c mice, 6-12 h post infestation, tick bite lesions are characterized histologically by strong innate reaction with predominance of neutrophils and mononuclear cells (Mbow et al., 1994). Similarly, an 84 kD *Haemaphysalis longicornis* protein induces an immediate hypersensitivity reaction as a type of inflammatory response (Mulenga et al., 2000). Also saliva of *Rhiphicephalus sanguineus* was shown to induce basophil and neutrophil infiltration (Szabo et al., 1995). These early alterations in tick bite lesions are comparable to our findings in paw edema showing an initial strong, transient innate immune response.

Studies have shown that tick saliva induces Th2 specific response throughout the feeding, showing higher IL-4, IL-5, IL-10 expression (Leboulle et al., 2002; Langhansova et al., 2015; Mejri and Brossard 2007). These responses increase as feeding continues (Langhansova et al., 2015). This facilitates transmission of pathogens such as *Rickettsia* spp., *Ehrlichia* spp., and *Borrelia burgdorferi* (Saraiva et al., 2014; Starkey et al., 2015; Labuda and Nuttall 2004). Also, the initial pro-inflammatory cytokines for these pathogens are beneficial for their proliferation and survival (Meddeb et al., 2016; Sahni et al., 2009; Lepidi et al., 2009). In our study, we suspect that the PI-

rTSPs induce the pro-inflammatory effect during the initial tick feeding process, hence possibly aiding in pathogen transmission. However, we were not able to show the predominant Th2 response by AI-rTSPs even though they suppress the PI-rTSPs induced pro-inflammatory response effect. This may be due to several reasons, first, being the development of Th2 response require longer than 48 h response, and second, the mRNA levels of cytokines that promote T cells migration may not be sensitive to actual cytokine detection. Thus, our idea was to detect the PI-rTSPs affecting the initial phase of feeding. This critical window of time for ticks to transmit pathogens and promoting modulatory effect on host response underlies the importance of tick removal as early as possible and hence control tick feeding.

In conclusion, PI rTSPs produce a strong innate immune response with possible infiltration of mononuclear cells, neutrophils, dendritic cells and up-regulation of chemokines and cytokines and could be recognized as biomarkers of acute phase of tick feeding. We suspect that tick saliva could weaken these responses and modulate the host towards Th2 responses. This could facilitate blood intake and transmission of pathogens. Thus, this study contributes to a deeper understanding of tick feeding physiology and identification of TSPs that are involved in mediating *A. americanum* tick's evasion of host defenses.

CHAPTER IV

EVALUATE PROTECTIVE EFFICACY OF PRO-INFLAMMATORY RTSPS AGAINST TICK FEEDING

Introduction

Work in our lab has described six *Amblyomma americanum* tick saliva insulin-like growth factor binding proteins-related proteins (*Aam*IGFBP-rP) (Mulenga et al., 2007, 2010, Kim et al, *in preparation*). Transcripts for three of these proteins *Aam*IGFBP-rP1, *Aam*IGFBP-rP6S, and *Aam*IGFBP-rP6L were originally identified among genes that were significantly up regulated in *A. americanum* ticks that were stimulated to start feeding on cattle (Mulenga et al., 2007). In subsequent studies *Aam*IGFBP-rP1, *Aam*IGFBP-rP6S, and *Aam*IGFBP-rP6L were demonstrated to be important in tick feeding physiology and general tick fitness as disruption of encoding transcripts using RNAi silencing resulted in reduced blood meal sizes, darkening and softness of the cuticle (Mulenga et al., 2013). In the same study, transcriptional analysis suggested these molecules were expressed in multiple tick organs (Mulenga et al., 2010), and in recent studies, were demonstrated present in the tick saliva proteome (Radulovic et al., 2015; Kim et al., *in preparation*). Although structurally, they are predicted to bind insulin-like growth factors, we showed that all three proteins, *Aam*IGFBP-rP1, *Aam*IGFBP-rP6S, and *Aam*IGFBP-rP6L, bound human insulin (Radulovic et al., 2015; Radulovic et al., *in preparation*). Results from chapters 2, 3 and 4 of this dissertation have provided further insight into the functional roles of these proteins as pro-inflammatory tick saliva proteins that enhanced macrophage phagocytosis. Collectively

these data from previous studies cited here (Mulenga et al., 2007; 2013; Radulovic et al., 2015 and *unpublished* Kim et al., *in preparation*) and data in this dissertation demonstrate that *Aam*IGFBP-rP1, *Aam*IGFBP-rP6S, and *Aam*IGFBP-rP6L are functionally important to tick feeding physiology. Thus there is likelihood that blocking functions of the three proteins could affect tick-feeding success. The goal of this fifth chapter was to determine immunogenicity and anti-tick vaccine efficacy of human embryo kidney (HEK) cell expressed r*Aam*IGFBP-rP1, r*Aam*IGFBP-rP6S, and r*Aam*IGFBP-rP6L against tick feeding in rabbits. The objective was to investigate if immunization with the cocktail of mammalian cell expressed r*Aam*IGFBPrP-1, r*Aam*IGFBP-rP6S r*Aam*IGFBP-rP6L (PI-rTSPs) was protective against *A. americanum* feeding success.

Commercialization of two vaccines against *Rhiphicephalus microplus* based on midgut antigens, Bm86 and Bm95 (Rodriguez et al., 1995; Willadsen et al., 1995; De la Feunte et al., 2000) gave credence to the concept of anti-tick immunization as a sustainable alternative tick control. The limitations of this vaccine such as effectiveness against a single tick specie and being limited to specific regions were quickly realized. The search for effective anti-tick vaccine antigens has continued such as an immune-dominant p29 *Haemaphysalis longicornis* that caused 50% mortality of larvae and nymphs (Mulenga et al., 1999), AAS19 which reduced adult *A. americanum* feeding efficiency by 50% (Kim et al., 2016), and a cement protein, 64P, that conferred protective immunity against *R. appendiculatus* as revealed by up to 48% and 70% mortality of nymph and adult ticks (Havlikova et al., 2009). In other studies a conserved

antigen, Subolesin, that are important in *Ixodes scapularis* innate immunity (Almazán et al., 2003), conferred protective immunity against *I. scapularis*, *A. americanum*, *R. microplus, R. annulatus* and *Dermacentor variabilis* (Almazán et al., 2005; 2010; Canales et al., 2009; De la Fuente et al., 2010) Another molecule called Ferritin 2, an iron storage protein, has been shown to reduce feeding, oviposition and fertility in *I. ricinus, R. microplus* and *R. annulatus* (Hajdusek et al., 2010). Although few in number, anti-tick vaccination studies against *A. americanum* are ongoing. In 2010, De la Fuente et al. immunized cattle against recombinant antigens encoding for threonyl-tRNA synthetase (2C9), 60S ribosomal proteins L13a and L13e and observed >30% protection against adults and nymphs, demonstrating feasibility of developing vaccines for control of lone star tick infestation (De la Fuente et al., 2010). Our lab recently demonstrated that rabbit immunity to a tick serpin AAS19, caused 60% of ticks fail to lay eggs and 50% reduction in engorgement weights (Kim et al., 2016). The observations that immunization of animals with single antigens confers partial protection have resulted in renewed focus to develop a vaccine that targets multiple tick antigens. In this fifth chapter of the dissertation, an attempt was made to evaluate the anti-tick vaccine efficacy of *A. americanum* pro-inflammation peptides that have been described in earlier chapters of this dissertation.

Materials and Methods

Expression of pro-inflammation (PI) recombinant (r) AamIGFBP-rP1, AamIGFBP-rP6 long, AamIGFBP-rP6 short in mammalian cells

Expression of PI-rTSPs (*AamIGFBP-rP1, AamIGFBP-rP6L, AamIGFBP-rP6S)*

were done in human embryonic kidney cells as described in chapter II. HEK-293F suspension cultures were grown to 1×10^6 viable cells/ml in 293 Freestyle medium (Thermo Scientific, Wilmington, DE) with shaking at 125 rpm in an incubator set to 8% CO_2 and 85% relative humidity. For transfection in suspension cultures, 100 μg recombinant plasmids and 100 μL of the transfection reagent, 293fectin (Thermo Scientific, Wilmington, DE) were incubated with ~1-2 mL Opti-MEM medium (Thermo Scientific, Wilmington, DE) separately. Following incubation, 293fectin and recombinant plasmids were combined and incubated for an additional 30 min and then added to a cell culture containing 1×10^8 HEK-293F cells. The cell suspension was harvested after 72 h and cell viability was routinely confirmed by trypan blue staining (<90% viability).

After verification of rTSP expression using western blotting analyses, cell lysates and the supernatant were incubated with the beads conjugated with the antibody to the FLAG tag (Sigma- Aldrich, St. Louis, MO) overnight at 4°C. The bound rTSPs were eluted using 0.1M Glycine buffer (pH 3.5) and neutralized with 1M Tris-HCl (pH 8). Following verification of protein elution, fractions were combined and dialyzed against Tris- HCl buffer (50 mM Tris, 150mM NaCl, pH 7.4) using spin filters (Pall Life Sciences ®, Port Washington, NY). Protein quantification was done using bicinchoninic acid assay (BCA assay) according to instructions provided by the manufacturer (Thermo Scientific, Wilmington, DE).

SDS-PAGE and western blotting analyses

Routine SDS-PAGE with Coommassie blue or silver staining and western blotting analyses was done to confirm purification. Proteins were resolved in 12.5% acrylamide/bis-acrylamide gels and stained with silver and coomassie stains. For western blotting analyses, resolved proteins were transferred to a PVDF (Polyvinylidene

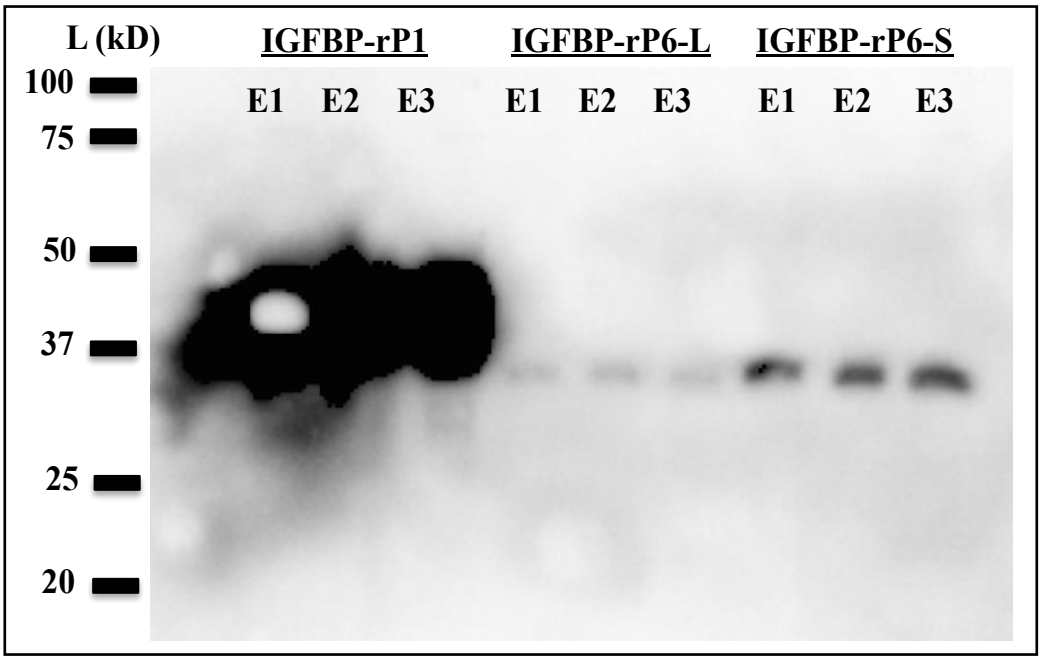


Figure 13. Expression and affinity purification of *Amblyomma americanum* IGFBP-rP1, IGFBP-rP6 long and IGFBP-rP short. Affinity purified recombinant tick saliva proteins were subjected to SDS-PAGE and western blot using antibody to flag tag. Left to right: Ladder (L), kD (kilodaltons), IGFBP-rP1 (tick insulin like growth factor binding protein 1), IGFBP-rP6-L (tick insulin like growth factor binding protein long), IGFBP-rP6-S (tick insulin like growth factor binding protein short), E (1-3): Elution from purifications.

difluoride) membrane using the Thermo-fisher western blot transfer system (Thermo Scientific, Wilmington, DE). Following transfer, membranes were blocked overnight at 4°C in 5% skim milk in PBS-Tween20. Subsequently membranes were washed and then incubated with anti-flag antibody conjugated with HRP (1:5000). The membranes were washed using PBS-0.05% Tween20 and proteins were confirmed using chemiluminescent substrate on X-ray films or using the Chemidoc MP gel doc (Biorad, Hercules, CA).

Results and Discussion

Insufficient expression and purification of rAamIGFBP-rP1, rAamIGFBP-rPL, AamIGFBP-rP6S for immunizations

The fifth chapter of this dissertation did proceed as intended. The limitation was that, except for r*Aam*IGFBP-rP1, efforts to produce sufficient amounts of r*Aam*IGFBP-rP6L and r*Aam*IGFBP-rP6S for immunization were unsuccessful (Fig.13). As shown in figure 13, the concentrated purifications confirmed expression as verified by western blot analyses using anti-body to Flag tag. The protein bands were observed at the expected molecular weight, with rTSPs r*Aam*IGFBP-rP1 showing intense bands following r*Aam*IGFBP-rP6L and r*Aam*IGFBP-rP6S. Despite positive expression and high sensitivity of western blot analyses, amounts of r*Aam*IGFBP-rP6L and r*Aam*IGFBP-rP6S were not sufficient to proceed with immunization.

Several possibilities could explain failures encountered in this fifth chapter. One possibility is that the transiently transfected genetic material can be lost by several environmental factors and cell divisions. Therefore, the inability of cells to produce

enough proteins due to high passage number of the cells may have contributed in loss of plasmid during cell division. Another possibility could be the requirement of increasing amounts of plasmid DNA for large scale expression. Similar to virus based systems that include an additional production step to generate sufficient amount of proteins for virus production (Condreay et al., 1999), it also becomes necessary to scale up the plasmid concentration for large scale expression in HEK expression system. Even though large scale expression was attempted by using increasing concentrations of plasmid, the conditions for large scale peptide production must be different compared to small scale expression.

As alternative strategies to obtain higher production concentrations of proteins, 293A cells were re-transfected and isolated from positive colonies. Fresh stocks of re-transformed bacteria containing expression plasmid were made and plasmid purification was done using ultra-pure water. The 293 freestyle cells (Kindly provided by Dr. Mwangi, Kansas State University, Manhattan, Kansas) were transfected with newly isolated plasmid. The expression culture was purified with new FLAG tag affinity beads. While the expression was consistently positive, I failed to purify enough recombinant proteins for immunization experiments. Failure to express high amount of recombinant IGFBPs prevented successful completion of the immunization and tick challenge experiments as outlined in my proposal. To attempt production of sufficient amounts of antigen, alternative strategies were developed. These include: (i) expression in stably transfected insect cells were developed, and (ii) expression in adherent 293 cells.

CHAPTER V

CONCLUSIONS

Summation of findings

The innate immune defense is the host's first defense mechanism that ticks encounter (Wikel 2013). Ticks regulate the macrophages for the maintenance of feeding lesions (Poole et al., 2013). Under this assumption, this dissertation focused on studying the effects of fifteen yeast- and insect cell- produced *Amblyomma americanum* and *Ixodes scapularis* recombinant tick saliva proteins (rTSP) on macrophage function. In chapter 2, preliminary analyses showed that out of fifteen rTSPs, five *A. americanum* rTSPs (here in referred to as pro-inflammation [PI] proteins) including Insulin-like growth factor-binding proteins-related proteins, *Aam*IGFBPrP-1, *Aam*IGFBP-rP6S, and *Aam*IGFBP-rP6L, Serine protease inhibitor (AAS8), tick carboxypeptidase inhibitor (*Aam*TCI) modulate macrophages by inducing expression of pro-inflammation cell surface co-stimulatory markers CD40, CD80 and CD86 and secretion of nitric oxide. These data were generated using an *in vitro* culture system using a macrophage cell line, RAW 264.7. *In vitro* results prompted additional recombinant protein production to verify the effect using mammalian-expressed proteins r*Aam*IGFBPrP-1, r*Aam*IGFBP-rP6S, r*Aam*IGFBP-rP6L, AAS8 and TCI. Interestingly, the response of macrophages was comparable to the preliminary analysis. I further confirmed that treatment-activated macrophages promoted the expression of pro-inflammatory cytokines including TNFα, IL-1, IL-6 and IL-10 induced by rTSPs. In contrast, two *A.*

americanum serine protease inhibitors, AAS27 and AAS41 (AI), reversed the pro-inflammatory response induced by LPS or PI-rTSPs.

I was curious to investigate if cell culture validated PI-rTSP and AI-rTSP were functional *in vivo*. Therefore, I investigated the role of PI-rTSPs and AI-rTSPs *in vivo* using a paw edema assay. Interestingly, cell culture results were validated *in vivo* as PI-rTSPs induced edema while AI-rTSP surpressed edema. Results showed that PI-rTSPs are positive regulators of cytokines TNFα, IL-1, IL-6 and chemokines CXCL1, CCL2, CCL3, CCL5 and CCL11 that were directly related to edema progression and inflammation. In contrast, AI- rTSPs are negative regulators of pro-inflammation markers including chemokines TNFα, CXCL1, CCL2 and CCL3.

Another interesting observation was that r*Aam*IGFBPrP-1, r*Aam*IGFBP-rP6S, and r*Aam*IGFBP-rP6L treatment of macrophages not only enhanced genes that are associated with phagocytosis, but also enhanced phagocytosis in macrophages. In the context of tick transmission of tick borne pathogens, the finding is particular significant. Several tick-borne pathogens have been shown to exploit tick proteins to establish infection in the mammalian host (Nuttall and Labuda 2008, Hovious et al., 2008; Labuda et al., 1993). Transmission of *Amblyomma* spp.-vectored tick-borne pathogens such as *Erhlichia chaffeensis* (Varela-Stokes 2007), *E. ruminantium* (Bekker et al., 2002), and the newly described tick-borne viruses, severe fever with thrombocytopenia virus (SFTSV) and heartland virus (HRTV) (Yun et al., 2014; Matsuno et al., 2017) target macrophages through phagocytosis for survival (Banajee et al., 2016, Ganta et al., 2009) and transmission. Could the observed enhancement of

phagocytosis suggest functional role(s) for r*Aam*IGFBPrP-1, r*Aam*IGFBP-rP6S, and r*Aam*IGFBP-rP6L in vector competence of *A. americanum*?

Another application or significance of the research is finding potentially attractive candidates for prophylactic applications. The field of medicine have evolved to take advantage of inflammatory mediators in treatment of inflammation-related diseases and vaccine development. The therapeutic use of nonspecific immune-modulatory molecules that boost host defenses have been used to treat chronic diseases (Yamashita et al., 2015). Based on my dataa, the bidirectional signaling presents new potential avenues for the development of therapeutic interventions targeting co-stimulatory and inflammatory molecules. As a result, deciphering the CD40/CD80/CD86 signaling pathway and the molecules that govern their expression is essential. In vaccine development, adjuvants made of recombinant proteins or ligands of immune cells potentiate the host response to infections. Pharmacological agents with anti-inflammatory properties are used to control adverse pathological conditions caused by inflammatory mediators (D'Elia et al., 2013). CD80 and CD86 have been identified as key regulators of immune activation, tolerance regulation and the skewing of T cell responses in disease models, such as graft-vs-host diseases, cancer, and autoimmune diseases (Salomon and Bluestone 2001). These therapeutic approaches are used to enhance the efficacy of antimicrobials in treatment of many diseases. Therefore, exploring the therapeutic potential of tick saliva proteins described here needs further investigation.

Future perspectives

First, although the dissertation achieved the majority of its objectives, there were some limitations. First, from the preliminary analysis, the *in vitro* assays utilized immortalized mouse cell lines, Raw macrophages. The advantage was the ease of culturing, determining macrophage-specific response and consistency within technical replicates. However, the immortalized cell lines may not mimic 100% of the primary cell function. However, the observation that cell culture data were validated using an *in vivo* assay provided credence to the cell culture-based findings in this dissertation. Second, I was unsuccessful in obtaining sufficient amount of rTSPs for experimental animal immunization and tick-feeding trials. One possible reason was the use of a transient expression system for obtaining high concentrations of rTSPs. The alternative strategy could be the expression of recombinant proteins in yeast or insect cell expression systems.

Overall, by combining functional and biological characterization, this dissertation provides additional insights into elucidating the pro- and anti-inflammatory function of three *A. americanum* IGFBPs and two serine protease inhibitors in tick feeding physiology. The observations that some of the recombinant *A. americanum* proteins investigated promoted inflammation and others down-regulated inflammatory markers was counter-intuitive, but not entirely novel. As ticks are expected to suppress inflammation to successfully feed and transmit pathogens, it is counter-intuitive for ticks to secrete proteins such as the 84 kDa in *Haemaphysalis longicornis* that induced immediate hypersensitivity in rabbits (Mulenga et al., 2000) and tick histamine release

factor which stimulated basophils to secrete an inflammation agonist, histamine (Mulenga et al., 2003). While transcriptional and gene expression analyses showed remarkable gene activation by PI-rTSPs and AI-rTSPs, further studies are required to understand the differential regulation between protein and transcripts. It would be of interest to identify the transcriptional factors involved for each protein in the context of pathways or signaling molecules described. Moreover, studies could be conducted to investigate the role of cytokines from T cells for interaction/binding of tick saliva proteins on these molecules. Some remaining questions to be resolved would be determining the TSP interactions with receptors of host macrophages, and detection of specific metabolic pathways and their role in pathogen transmission. Additionally, it would be of interest to study the adjuvanticity and anti-tick vaccine efficacy of pro-inflammatory rTSPs against ticks and the transmission of tick-borne diseases. Whether or not their inclusion in vaccines can result in protection from tick feeding and/or pathogen transmission remains to be investigated.

REFERENCES

Abbas, R.Z., Zaman, M.A., Colwell, D.D., Gilleard, J., Iqbal, Z., 2014. Acaricide resistance in cattle ticks and approaches to its management: the state of play. Vet. Parasitol. 203, 6-20.

Agner, K., 1941. Verdoperoxidase. Wiley Online Library.

Ahantarig, A., Ruzek, D., Vancova, M., Janowitz, A., St'astna, H., Tesarova, M., Grubhoffer, L., 2009. Tick-borne encephalitis virus infection of cultured mouse macrophages. Intervirology 52, 283-290.

Ahmad, M.F., Ahmad, S.M., Keservani, R.K., Sharma, A.K., 2016. Anti-inflammatory Activity of Tuber Extracts of Solanum tuberosum in Male Albino Rats. National Academy Science Letters 39, 421-425.

Ahn, Y.T., Huang, B., McPherson, L., Clayberger, C., Krensky, A.M., 2007. Dynamic interplay of transcriptional machinery and chromatin regulates "late" expression of the chemokine RANTES in T lymphocytes. Mol. Cell. Biol. 27, 253-266.

Alarcon- Chaidez, F., Boppana, V., Hagymasi, A., Adler, A., Wikel, S., 2009. A novel sphingomyelinase- like enzyme in Ixodes scapularis tick saliva drives host CD4 T cells to express IL- 4. Parasite Immunol. 31, 210-219.

Aljamali, M., Hern, L., Kupfer, D., Downard, S., So, S., Roe, B., Sauer, J., Essenberg, R., 2009. Transcriptome analysis of the salivary glands of the female tick Amblyomma americanum (Acari: Ixodidae). Insect Mol. Biol. 18, 129-154.

Almazán, C., Kocan, K.M., Bergman, D.K., Garcia-Garcia, J.C., Blouin, E.F., de la Fuente, J., 2003. Identification of protective antigens for the control of Ixodes scapularis infestations using cDNA expression library immunization. Vaccine 21, 1492-1501.

Almazán, C., Kocan, K.M., Blouin, E.F., de la Fuente, J., 2005. Vaccination with recombinant tick antigens for the control of Ixodes scapularis adult infestations. Vaccine 23, 5294-5298.

Almazán, C., Lagunes, R., Villar, M., Canales, M., Rosario-Cruz, R., Jongejan, F., de la Fuente, J., 2010. Identification and characterization of Rhipicephalus (Boophilus) microplus candidate protective antigens for the control of cattle tick infestations. Parasitol. Res. 106, 471-479.

Amdekar, S., Roy, P., Singh, V., Kumar, A., Singh, R., Sharma, P., 2012. Anti-inflammatory activity of lactobacillus on carrageenan-induced paw edema in male wistar rats. International journal of inflammation 2012.

Annen, K., Friedman, K., Eshoa, C., Horowitz, M., Gottschall, J., Straus, T., 2012. Two cases of transfusion-transmitted Anaplasma phagocytophilum. Am. J. Clin. Pathol. 137, 562-565.

Atkinson, B., Chamberlain, J., Jameson, L.J., Logue, C.H., Lewis, J., Belobrova, E.A., Valikhodzhaeva, M., Mullojonova, M., Tishkova, F.H., Hewson, R., 2013. Identification and analysis of Crimean-Congo hemorrhagic fever virus from human sera in Tajikistan. International Journal of Infectious Diseases 17, e1031-e1037.

Awumbila, B., 1996. Acaricides in tick control in Ghana and methods of application. Trop. Anim. Health Prod. 28, 50S-52S.

Banajee, K.H., Embers, M.E., Langohr, I.M., Doyle, L.A., Hasenkampf, N.R., Macaluso, K.R., 2015. Amblyomma maculatum Feeding Augments Rickettsia parkeri Infection in a Rhesus Macaque Model: A Pilot Study. PloS one 10, e0135175.

Banajee, K., Verhoeve, V., Harris, E., Macaluso, K., 2016. Effect of Amblyomma maculatum (Acari: Ixodidae) Saliva on the Acute Cutaneous Immune Response to Rickettsia parkeri Infection in a Murine Model. J. Med. Entomol. 53, 1252-1260.

Barratt-Due, A., Thorgersen, E.B., Lindstad, J.K., Pharo, A., Lissina, O., Lambris, J.D., Nunn, M.A., Mollnes, T.E., 2011. Ornithodoros moubata complement inhibitor is an equally effective C5 inhibitor in pigs and humans. J. Immunol. 187, 4913-4919.

Bekker, C.P., de Vos, S., Taoufik, A., Sparagano, O.A., Jongejan, F., 2002. Simultaneous detection of Anaplasma and Ehrlichia species in ruminants and detection of Ehrlichiaruminantium in Amblyomma variegatum ticks by reverse line blot hybridization. Vet. Microbiol. 89, 223-238.

Benly, P., 2015. Role of histamine in acute inflammation. Journal of Pharmaceutical Sciences and Research 7, 373-376.

Bernard, Q., Wang, Z., Di Nardo, A., Boulanger, N., 2017. Interaction of primary mast cells with Borrelia burgdorferi (sensu stricto): role in transmission and dissemination in C57BL/6 mice. Parasites & vectors 10, 313.

Blakesley, R.W., Boezi, J.A., 1977. A new staining technique for proteins in polyacrylamide gels using Coomassie Brilliant Blue G250. Anal. Biochem. 82, 580-582.

Blum, H., Beier, H., Gross, H.J., 1987. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis 8, 93-99.

Boppana, D.K., Dhinakar, R., John, L., Wikel, S.K., Latha, B., Gomathinayagam, S., 2004. In vivo immunomodulatory effects of ixodid ticks on ovine circulating T- and B-lymphocytes. Parasite Immunol. 26, 83-93.

Boppana, D., Wikel, S., Raj, D., Manohar, M., Lalitha, J., 2005. Cellular infiltration at skin lesions and draining lymph nodes of sheep infested with adult Hyalomma anatolicum anatolicum ticks. Parasitology 131, 657-667.

Bowman, A., Coons, L., Needham, G., Sauer, J., 1997. Tick saliva: recent advances and implications for vector competence. Med. Vet. Entomol. 11, 277-285.

Brake, D.K., de León, Adalberto A Pérez, 2012. Immunoregulation of bovine macrophages by factors in the salivary glands of Rhipicephalus microplus. Parasites & vectors 5, 38.

Brake, D.K., Wikel, S.K., Tidwell, J.P., de León, Adalberto A Pérez, 2010. Rhipicephalus microplus salivary gland molecules induce differential CD86 expression in murine macrophages. Parasites & vectors 3, 103.

Breitschwerdt, E.B., Maggi, R.G., Chomel, B.B., Lappin, M.R., 2010. Bartonellosis: an emerging infectious disease of zoonotic importance to animals and human beings. Journal of Veterinary Emergency and Critical Care 20, 8-30.

Brites-Neto, J., Duarte, K.M.R., Martins, T.F., 2015. Tick-borne infections in human and animal population worldwide. Veterinary world 8, 301.

Brito, L.G., Barbieri, F.S., Rocha, R.B., Oliveira, M.C., Ribeiro, E.S., 2011. Evaluation of the Efficacy of Acaricides Used to Control the Cattle Tick, Rhipicephalus microplus, in Dairy Herds Raised in the Brazilian Southwestern Amazon. Vet. Med. Int. 2011, 806093.

Brodskyn, C.I., DeKrey, G.K., Titus, R.G., 2001. Influence of costimulatory molecules on immune response to Leishmania major by human cells in vitro. Infect. Immun. 69, 665-672.

Bullard, R., Allen, P., Chao, C., Douglas, J., Das, P., Morgan, S.E., Ching, W., Karim, S., 2016. Structural characterization of tick cement cones collected from in vivo and artificial membrane blood-fed Lone Star ticks (Amblyomma americanum). Ticks and tick-borne diseases 7, 880-892.

Burgdorfer, W., Barbour, A.G., Hayes, S.F., Benach, J.L., Grunwaldt, E., Davis, J.P., 1982. Lyme disease-a tick-borne spirochetosis? Science 216, 1317-1319.

Callow, L., Dalgliesh, R., De Vos, A., 1997. Development of effective living vaccines against bovine babesiosis—the longest field trial? Int. J. Parasitol. 27, 747-767.

Canales, M., Naranjo, V., Almazán, C., Molina, R., Tsuruta, S.A., Szabó, M.P., Manzano-Roman, R., de la Lastra, José M Pérez, Kocan, K.M., Jiménez, M.I., 2009. Conservation and immunogenicity of the mosquito ortholog of the tick-protective antigen, subolesin. Parasitol. Res. 105, 97-111.

Carr, M.W., Roth, S.J., Luther, E., Rose, S.S., Springer, T.A., 1994. Monocyte chemoattractant protein 1 acts as a T-lymphocyte chemoattractant. Proc. Natl. Acad. Sci. U. S. A. 91, 3652-3656.

Carreno, B.M., Collins, M., 2002. The B7 family of ligands and its receptors: new pathways for costimulation and inhibition of immune responses. Annu. Rev. Immunol. 20, 29-53.

Carvalho, W.A., Franzin, A.M., Abatepaulo, A.R.R., de Oliveira, Carlo José Freire, Moré, D.D., da Silva, J.S., Ferreira, B.R., de Miranda Santos, Isabel K Ferreira, 2010. Modulation of cutaneous inflammation induced by ticks in contrasting phenotypes of infestation in bovines. Vet. Parasitol. 167, 260-273.

Carvalho-Costa, T.M., Mendes, M.T., da Silva, M.V., da Costa, T.A., Tiburcio, M.G.S., Anhê, Ana Carolina Borella Marfil, Rodrigues, V., Oliveira, C.J.F., 2015. Immunosuppressive effects of Amblyomma cajennense tick saliva on murine bone marrow-derived dendritic cells. Parasites & vectors 8, 22.

Castagnolli, K.C., Ferreira, B.R., Franzin, A.M., De Castro, M.B., Szabó, M.P.J., 2008. Effect of Amblyomma cajennense ticks on the immune response of BALB/c mice and horses. Ann. N. Y. Acad. Sci. 1149, 230-234.

Cavassani, K.A., Aliberti, J.C., Dias, A.R., Silva, J.S., Ferreira, B.R., 2005. Tick saliva inhibits differentiation, maturation and function of murine bone- marrow- derived dendritic cells. Immunology 114, 235-245.

Chen, G., Severo, M.S., Sohail, M., Sakhon, O.S., Wikel, S.K., Kotsyfakis, M., Pedra, J.H., 2012. Ixodes scapularis saliva mitigates inflammatory cytokine secretion during Anaplasma phagocytophilum stimulation of immune cells. Parasites & vectors 5, 229.

Chmelar, J., Oliveira, C.J., Rezacova, P., Francischetti, I.M., Kovarova, Z., Pejler, G., Kopacek, P., Ribeiro, J.M., Mares, M., Kopecky, J., Kotsyfakis, M., 2011. A tick

salivary protein targets cathepsin G and chymase and inhibits host inflammation and platelet aggregation. Blood 117, 736-744.

Clark, R.A., Leidal, K.G., Pearson, D.W., Nauseef, W.M., 1987. NADPH oxidase of human neutrophils. Subcellular localization and characterization of an arachidonate-activatable superoxide-generating system. J. Biol. Chem. 262, 4065-4074.

Coles, T.B., Dryden, M.W., 2014. Insecticide/acaricide resistance in fleas and ticks infesting dogs and cats. Parasites & vectors 7, 8.

Condreay, J.P., Witherspoon, S.M., Clay, W.C., Kost, T.A., 1999. Transient and stable gene expression in mammalian cells transduced with a recombinant baculovirus vector. Proc. Natl. Acad. Sci. U. S. A. 96, 127-132.

Dann, S.M., Spehlmann, M.E., Hammond, D.C., Iimura, M., Hase, K., Choi, L.J., Hanson, E., Eckmann, L., 2008. IL-6-dependent mucosal protection prevents establishment of a microbial niche for attaching/effacing lesion-forming enteric bacterial pathogens. J. Immunol. 180, 6816-6826.

Dantas-Torres, F., 2015. Climate change, biodiversity, ticks and tick-borne diseases: The butterfly effect. International Journal for Parasitology: Parasites and Wildlife 4, 452-461.

Dantas-Torres, F., Chomel, B.B., Otranto, D., 2012. Ticks and tick-borne diseases: a One Health perspective. Trends Parasitol. 28, 437-446.

Dash, Y., Maxwell, S., Rajan, T., Wikel, S., 2005. Murine extramedullary erythropoiesis induced by tick infestation. Annals of Tropical Medicine & Parasitology 99, 518-531.

Davis, M.J., Tsang, T.M., Qiu, Y., Dayrit, J.K., Freij, J.B., Huffnagle, G.B., Olszewski, M.A., 2013. Macrophage M1/M2 polarization dynamically adapts to changes in cytokine microenvironments in Cryptococcus neoformans infection. MBio 4, e00264-13.

De la Fuente, J., Almazán, C., Canales, M., de la Lastra, José Manuel Pérez, Kocan, K.M., Willadsen, P., 2007. A ten-year review of commercial vaccine performance for control of tick infestations on cattle. Animal Health Research Reviews 8, 23-28.

De la Fuente, J., Manzano-Roman, R., Naranjo, V., Kocan, K.M., Zivkovic, Z., Blouin, E.F., Canales, M., Almazán, C., Galindo, R.C., Step, D.L., 2010. Identification of protective antigens by RNA interference for control of the lone star tick, Amblyomma americanum. Vaccine 28, 1786-1795.

De la Fuente, J., Merino, O., 2013. Vaccinomics, the new road to tick vaccines. Vaccine 31, 5923-5929.

De la Fuente, J., Moreno-Cid, J.A., Canales, M., Villar, M., de la Lastra, José M Pérez, Kocan, K.M., Galindo, R.C., Almazán, C., Blouin, E.F., 2011. Targeting arthropod subolesin/akirin for the development of a universal vaccine for control of vector infestations and pathogen transmission. Vet. Parasitol. 181, 17-22.

De la Fuente, J., Rodriguez, M., Montero, C., Redondo, M., Garcia-Garcia, J.C., Mendez, L., Serrano, E., Valdes, M., Enriquez, A., Canales, M., 1999. Vaccination against ticks (Boophilus spp.): the experience with Bm86-based vaccine Gavac^{TM}. Genet. Anal. : Biomol. Eng. 15, 143-148.

De La Fuente, J., Kocan, K.M., Contreras, M., 2015. Prevention and control strategies for ticks and pathogen transmission. Rev. Sci. Tech. 34, 249-264.

De Vos, S., Zeinstra, L., Taoufik, A., Willadsen, P., Jongejan, F., 2001. Evidence for the utility of the Bm86 antigen from Boophilus microplus in vaccination against other tick species. Experimental and Applied Acarology 25, 245-261.

De la Fuente, J., RodrÍguez, M., Garcia- Gari, J.C., 2000. Immunological control of ticks through vaccination with Boophilus microplus gut antigens. Ann. N. Y. Acad. Sci. 916, 617-621.

D'Elia, R.V., Harrison, K., Oyston, P.C., Lukaszewski, R.A., Clark, G.C., 2013. Targeting the "cytokine storm" for therapeutic benefit. Clin. Vaccine Immunol. 20, 319-327.

Deng, B., Wehling-Henricks, M., Villalta, S.A., Wang, Y., Tidball, J.G., 2012. IL-10 triggers changes in macrophage phenotype that promote muscle growth and regeneration. J. Immunol. 189, 3669-3680.

Deruaz, M., Frauenschuh, A., Alessandri, A.L., Dias, J.M., Coelho, F.M., Russo, R.C., Ferreira, B.R., Graham, G.J., Shaw, J.P., Wells, T.N., Teixeira, M.M., Power, C.A., Proudfoot, A.E., 2008. Ticks produce highly selective chemokine binding proteins with antiinflammatory activity. J. Exp. Med. 205, 2019-2031.

Díaz-Martín, V., Manzano-Román, R., Valero, L., Oleaga, A., Encinas-Grandes, A., Pérez-Sánchez, R., 2013. An insight into the proteome of the saliva of the argasid tick Ornithodoros moubata reveals important differences in saliva protein composition between the sexes. Journal of proteomics 80, 216-235.

Donlon, T., Krensky, A., Wallace, M.R., Collins, F.S., Lovett, M., Clayberger, C., 1990. Localization of a human T-cell-specific gene, RANTES (D17S136E), to chromosome 17q11. 2-q12. Genomics 6, 548-553.

Eberlein, J., Nguyen, T.T., Victorino, F., Golden-Mason, L., Rosen, H.R., Homann, D., 2010. Comprehensive assessment of chemokine expression profiles by flow cytometry. J. Clin. Invest. 120, 907-923.

Estrada-Peña, A., Jongejan, F., 1999. Ticks feeding on humans: a review of records on human-biting Ixodoidea with special reference to pathogen transmission. Exp. Appl. Acarol. 23, 685-715.

Ewing, S., Dawson, J., Panciera, R., Mathew, J., Pratt, K., Katavolos, P., Telford III, S., 1997. Dogs infected with a human granulocytotropic Ehrlichia spp.(Rickettsiales: Ehrlichieae). J. Med. Entomol. 34, 710-718.

Ewing, S.A., Panciera, R.J., 2003. American canine hepatozoonosis. Clin. Microbiol. Rev. 16, 688-697.

Farkas, E., Süle, Z., Tóth-Szűki, V., Mátyás, A., Antal, P., Farkas, I.G., Mihály, A., Bari, F., 2006. Tumor necrosis factor-alpha increases cerebral blood flow and ultrastructural capillary damage through the release of nitric oxide in the rat brain. Microvasc. Res. 72, 113-119.

Fernando, M.R., Reyes, J.L., Iannuzzi, J., Leung, G., McKay, D.M., 2014. The pro-inflammatory cytokine, interleukin-6, enhances the polarization of alternatively activated macrophages. PLoS One 9, e94188.

Ferreira, B.R., Szabó, M.J., Cavassani, K.A., Bechara, G.H., Silva, J.S., 2003. Antigens from Rhipicephalus sanguineus ticks elicit potent cell-mediated immune responses in resistant but not in susceptible animals. Vet. Parasitol. 115, 35-48.

Fialová, A., Cimburek, Z., Iezzi, G., Kopecký, J., 2010. Ixodes ricinus tick saliva modulates tick-borne encephalitis virus infection of dendritic cells. Microb. Infect. 12, 580-585.

Francischetti, I.M., Meng, Z., Mans, B.J., Gudderra, N., Hall, M., Veenstra, T.D., Pham, V.M., Kotsyfakis, M., Ribeiro, J.M., 2008. An insight into the salivary transcriptome and proteome of the soft tick and vector of epizootic bovine abortion, Ornithodoros coriaceus. Journal of proteomics 71, 493-512.

Francischetti, I.M., Valenzuela, J.G., Andersen, J.F., Mather, T.N., Ribeiro, J.M., 2002. Ixolaris, a novel recombinant tissue factor pathway inhibitor (TFPI) from the salivary gland of the tick, Ixodes scapularis: identification of factor X and factor Xa as scaffolds for the inhibition of factor VIIa/tissue factor complex. Blood 99, 3602-3612.

Franco, P.F., Silva, N.C., do Vale, V.F., Abreu, J.F., Santos, V.C., Gontijo, N.F., Valenzuela, J.G., Pereira, M.H., Sant'Anna, M.R., Gomes, A.P., 2016. Inhibition of the classical pathway of the complement system by saliva of Amblyomma cajennense (Acari: Ixodidae). Exp. Parasitol. 164, 91-96.

Frauenschuh, A., Power, C.A., Deruaz, M., Ferreira, B.R., Silva, J.S., Teixeira, M.M., Dias, J.M., Martin, T., Wells, T.N., Proudfoot, A.E., 2007. Molecular cloning and characterization of a highly selective chemokine-binding protein from the tick Rhipicephalus sanguineus. J. Biol. Chem. 282, 27250-27258.

Freire, M.O., Van Dyke, T.E., 2013. Natural resolution of inflammation. Periodontol. 2000 63, 149-164.

Fujiwara, N., Kobayashi, K., 2005. Macrophages in inflammation. Current Drug Targets-Inflammation & Allergy 4, 281-286.

Fyumagwa, R.D., Runyoro, V., Horak, I.G., Hoare, R., 2007. Ecology and control of ticks as disease vectors in wildlife of the Ngorongoro Crater, Tanzania. S. Afr. J. Wildl. Res. 37, 79-90.

Galaï, Y., Canales, M., Saïd, M.B., Gharbi, M., Mhadhbi, M., Jedidi, M., de La Fuente, J., Darghouth, M., 2012. Efficacy of Hyalomma scupense (Hd86) antigen against Hyalomma excavatum and H. scupense tick infestations in cattle. Vaccine 30, 7084-7089.

Ganta, R.R., Peddireddi, L., Seo, G.M., Dedonder, S.E., Cheng, C., Chapes, S.K., 2009. Molecular characterization of Ehrlichia interactions with tick cells and macrophages. Front. Biosci. (Landmark Ed) 14, 3259-3273.

Garcia, G.R., Gardinassi, L.G., Ribeiro, J.M., Anatriello, E., Ferreira, B.R., Moreira, H.N.S., Mafra, C., Martins, M.M., Szabó, M.P.J., de Miranda-Santos, Isabel Kinney Ferreira, 2014. The sialotranscriptome of Amblyomma triste, Amblyomma parvum and Amblyomma cajennense ticks, uncovered by 454-based RNA-seq. Parasites & vectors 7, 430.

Getz, G.S., 2005. Thematic review series: the immune system and atherogenesis. Bridging the innate and adaptive immune systems. J. Lipid Res. 46, 619-622.

Glatz, M., Means, T., Haas, J., Steere, A.C., Müllegger, R.R., 2017. Characterization of the early local immune response to Ixodes ricinus tick bites in human skin. Exp. Dermatol. 26, 263-269.

Gong, D., Shi, W., Yi, S., Chen, H., Groffen, J., Heisterkamp, N., 2012. TGFβ signaling plays a critical role in promoting alternative macrophage activation. BMC immunology 13, 31.

Graf, J., Gogolewski, R., Leach-Bing, N., Sabatini, G., Molento, M., Bordin, E., Arantes, G., 2004. Tick control: an industry point of view. Parasitology 129, S427-S442.

Greene, C.E., Burgdorfer, W., Cavagnolo, R., Philip, R.N., Peacock, M.G., 1985. Rocky Mountain spotted fever in dogs and its differentiation from canine ehrlichiosis. J. Am. Vet. Med. Assoc. 186, 465-472.

Grigor'eva, L.A., 2001. Histopathologic changes of bird skin in feeding places of ticks of the genus Ixodes (Acari: Ixodidae). Parazitologiia 35, 490-495.

Guermonprez, P., Valladeau, J., Zitvogel, L., Théry, C., Amigorena, S., 2002. Antigen presentation and T cell stimulation by dendritic cells. Annu. Rev. Immunol. 20, 621-667.

Guerrero, F.D., Nene, V.M., George, J.E., Barker, S.C., Willadsen, P., 2006. Sequencing a new target genome: the Boophilus microplus (Acari: Ixodidae) genome project. J. Med. Entomol. 43, 9-16.

Guerrero, F.D., Lovis, L., Martins, J.R., 2012. Acaricide resistance mechanisms in Rhipicephalus (Boophilus) microplus. Revista Brasileira de Parasitologia Veterinária 21, 1-6.

Guglielmone, A.A., Robbins, R.G., Apanaskevich, D.A., Petney, T.N., Estrada-Peña, A., Shao, R., Barker, S.C., 2010. The Argasidae, Ixodidae and Nuttalliellidae (Acari: Ixodida) of the world: a list of valid species names.

Gulia-Nuss, M., Nuss, A.B., Meyer, J.M., Sonenshine, D.E., Roe, R.M., Waterhouse, R.M., Sattelle, D.B., de La Fuente, J., Ribeiro, J.M., Megy, K., 2016. Genomic insights into the Ixodes scapularis tick vector of Lyme disease. Nature communications 7, 10507.

Guma, M., Ronacher, L., Liu- Bryan, R., Takai, S., Karin, M., Corr, M., 2009. Caspase 1–independent activation of interleukin- 1β in neutrophil- predominant inflammation. Arthritis & Rheumatology 60, 3642-3650.

Guo, X., Booth, C.J., Paley, M.A., Wang, X., DePonte, K., Fikrig, E., Narasimhan, S., Montgomery, R.R., 2009. Inhibition of neutrophil function by two tick salivary proteins. Infect. Immun. 77, 2320-2329.

Hajdusek, O., Almazán, C., Loosova, G., Villar, M., Canales, M., Grubhoffer, L., Kopacek, P., De la Fuente, J., 2010. Characterization of ferritin 2 for the control of tick infestations. Vaccine 28, 2993-2998.

Hajnicka, V., Vančová, I., Kocakova, P., Slovak, M., Gašperík, J., Slavikova, M., Hails, R., Labuda, M., Nuttall, P., 2005. Manipulation of host cytokine network by ticks: a potential gateway for pathogen transmission. Parasitology 130, 333-342.

Hannier, S., Liversidge, J., Sternberg, J.M., Bowman, A.S., 2004. Characterization of the B- cell inhibitory protein factor in Ixodes ricinus tick saliva: a potential role in enhanced Borrelia burgdoferi transmission. Immunology 113, 401-408.

Harper, G., Riddles, P., Waltisbuhl, D., Wright, I., 1994. Applications of recombinant DNA technologies in the development of a vaccine: examples in the development of a vaccine against Babesia. Applications of recombinant DNA technologies in the development of a vaccine: examples in the development of a vaccine against Babesia. , 161-169.

Harrison, J.E., Schultz, J., 1976. Studies on the chlorinating activity of myeloperoxidase. J. Biol. Chem. 251, 1371-1374.

Hartelt, K., Wurst, E., Collatz, J., Zimmermann, G., Kleespies, R.G., Oehme, R.M., Kimmig, P., Steidle, J.L., Mackenstedt, U., 2008. Biological control of the tick Ixodes ricinus with entomopathogenic fungi and nematodes: Preliminary results from laboratory experiments. International Journal of Medical Microbiology 298, 314-320.

Hassimotto, N.M., Moreira, V., do Nascimento, N.G., Souto, P.C., Teixeira, C., Lajolo, F.M., 2013. Inhibition of carrageenan-induced acute inflammation in mice by oral administration of anthocyanin mixture from wild mulberry and cyanidin-3-glucoside. Biomed. Res. Int. 2013, 146716.

Havlíková, S., Roller, L., Koči, J., Trimnell, A.R., Kazimírová, M., Klempa, B., Nuttall, P.A., 2009. Functional role of 64P, the candidate transmission-blocking vaccine antigen from the tick, Rhipicephalus appendiculatus. Int. J. Parasitol. 39, 1485-1494.

Heinze, D.M., Carmical, J.R., Aronson, J.F., Thangamani, S., 2012. Early immunologic events at the tick-host interface. PloS one 7, e47301.

Heinze, D.M., Wikel, S.K., Thangamani, S., Alarcon-Chaidez, F.J., 2012. Transcriptional profiling of the murine cutaneous response during initial and subsequent infestations with Ixodes scapularis nymphs. Parasites & vectors 5, 26.

Hellman, P., Eriksson, H., 2007. Early activation markers of human peripheral dendritic cells. Hum. Immunol. 68, 324-333.

Hermance, M.E., Thangamani, S., 2015. Tick Saliva Enhances Powassan Virus Transmission to the Host, Influencing Its Dissemination and the Course of Disease. J. Virol. 89, 7852-7860.

Hill, C.A., Wikel, S.K., 2005. The Ixodes scapularis Genome Project: an opportunity for advancing tick research. Trends Parasitol. 21, 151-153.

Hodgson, J.L., 1992. Biology and transmission of Babesia bigemina in Boophilus microplus. Ann. N. Y. Acad. Sci. 653, 42-51.

Holbrook, A., Anthony, D., Johnson, A., 1968. Observations on the development of Babesia caballi (Nuttall) in the tropical horse tick Dermacentor nitens Neumann. J. Eukaryot. Microbiol. 15, 391-396.

Holdsworth, P., 2005. History of ectoparasiticide use in Australian livestock production. Ectoparasiticide use in contemporary Australian livestock production.Avcare Limited, Canberra , 7-18.

Horka, H., Staudt, V., Klein, M., Taube, C., Reuter, S., Dehzad, N., Andersen, J.F., Kopecky, J., Schild, H., Kotsyfakis, M., Hoffmann, M., Gerlitzki, B., Stassen, M., Bopp, T., Schmitt, E., 2012. The tick salivary protein sialostatin L inhibits the Th9-derived production of the asthma-promoting cytokine IL-9 and is effective in the prevention of experimental asthma. J. Immunol. 188, 2669-2676.

Hourcade, D.E., Akk, A.M., Mitchell, L.M., Zhou, H., Hauhart, R., Pham, C.T., 2016. Anti-complement activity of the Ixodes scapularis salivary protein Salp20. Mol. Immunol. 69, 62-69.

Hovius, J.W., Schuijt, T.J., de Groot, K.A., Roelofs, J.J., Oei, G., Marquart, J., de Beer, R., van't Veer, C., van der Poll, T., Ramamoorthi, N., 2008. Preferential protection of Borrelia burgdorferi sensu stricto by a Salp 15 homologue in Ixodes ricinus saliva. J. Infect. Dis. 198, 1189-1197.

Hovius, J.W., de Jong, Marein AW P, den Dunnen, J., Litjens, M., Fikrig, E., van der Poll, T., Gringhuis, S.I., Geijtenbeek, T.B., 2008. Salp15 binding to DC-SIGN inhibits cytokine expression by impairing both nucleosome remodeling and mRNA stabilization. PLoS pathogens 4, e31.

Ibelli, A.M., Kim, T.K., Hill, C.C., Lewis, L.A., Bakshi, M., Miller, S., Porter, L., Mulenga, A., 2014. A blood meal-induced Ixodes scapularis tick saliva serpin inhibits trypsin and thrombin, and interferes with platelet aggregation and blood clotting. Int. J. Parasitol. 44, 369-379.

Iida, N., Grotendorst, G.R., 1990. Cloning and sequencing of a new gro transcript from activated human monocytes: expression in leukocytes and wound tissue. Mol. Cell. Biol. 10, 5596-5599.

Inokuma, H., Aita, T., Ohno, K., Onishi, T., 1998. Effects of infestation by Rhipicephalus sanguineus on lymphocyte blastogenic responses to mitogens in dogs. Journal of veterinary medical science 60, 1013-1016.

Jaja-Chimedza, A., Graf, B.L., Simmler, C., Kim, Y., Kuhn, P., Pauli, G.F., Raskin, I., 2017. Biochemical characterization and anti-inflammatory properties of an isothiocyanate-enriched moringa (Moringa oleifera) seed extract. PloS one 12, e0182658.

Jiao, X., Fan, Z., Li, Y., Tang, Y., Ke, C., 2015. Clinical and laboratory features parameters of human granulocytic anaplasmosis (HGA) in patients admitted to hospital in Guangdong Province, China. Trop. Doct. 45, 209-213.

Johnson, R.C., Schmid, G.P., Hyde, F.W., Steigerwalt, A., Brenner, D.J., 1984. Borrelia burgdorferi sp. nov.: etiologic agent of Lyme disease. Int. J. Syst. Evol. Microbiol. 34, 496-497.

Jongejan, F., Uilenberg, G., 2004. The global importance of ticks. Parasitology 129, S3-S14.

Juncadella, I.J., Garg, R., Ananthnarayanan, S.K., Yengo, C.M., Anguita, J., 2007. T-cell signaling pathways inhibited by the tick saliva immunosuppressor, Salp15. FEMS Immunology & Medical Microbiology 49, 433-438.

Karim, S., Singh, P., Ribeiro, J.M., 2011. A deep insight into the sialotranscriptome of the gulf coast tick, Amblyomma maculatum. PloS one 6, e28525.

Kazimírová, M., 2007. Bioactive compounds in ticks acting on host thrombohemostasis. Thrombohemostatic disease research , 95-113.

Kazimírová, M., Jančinová, V., Petríková, M., Takáč, P., Labuda, M., Nosál, R., 2003. An inhibitor of thrombin-stimulated blood platelet aggregation from the salivary glands of the hard tick Amblyomma variegatum (Acari: Ixodidae), in Anonymous Ticks and Tick-Borne Pathogens. Springer, pp. 97-105.

Kazimirova, M., Stibraniova, I., 2013. Tick salivary compounds: their role in modulation of host defences and pathogen transmission. Front. Cell. Infect. Microbiol. 3, 43.

Keesing, F., Allan, B.F., Young, T.P., Ostfeld, R.S., 2013. Effects of wildlife and cattle on tick abundance in central Kenya. Ecol. Appl. 23, 1410-1418.

Keirans, J.E., Clifford, C.M., Hoogstraal, H., Easton, E.R., 1976. Discovery of nuttalliella namaqua bedford (acarina: Ixodoidea: Nuttalliellidae) in tanzania and redescription of the female based on scanning electron microcopy. Ann. Entomol. Soc. Am. 69, 926-932.

Kerber, C.E., Labruna, M.B., Ferreira, F., De Waal, D.T., Knowles, D.P., Gennari, S.M., 2009. Prevalence of equine Piroplasmosis and its association with tick infestation in the State of São Paulo, Brazil. Revista Brasileira de Parasitologia Veterinária 18, 1-8.

Kern, A., Liu, K., Mansbridge, J., 2001. Modification of fibroblast γ-interferon responses by extracellular matrix. J. Invest. Dermatol. 117, 112-118.

Khan, N., Gowthaman, U., Pahari, S., Agrewala, J.N., 2012. Manipulation of costimulatory molecules by intracellular pathogens: veni, vidi, vici!! PLoS pathogens 8, e1002676.

Kim, T.K., Radulovic, Z., Mulenga, A., 2016. Target validation of highly conserved Amblyomma americanum tick saliva serine protease inhibitor 19. Ticks and tick-borne diseases 7, 405-414.

Kim, T.K., Ibelli, A.M.G., Mulenga, A., 2015a. Amblyomma americanum tick calreticulin binds C1q but does not inhibit activation of the classical complement cascade. Ticks and tick-borne diseases 6, 91-101.

Kim, T.K., Tirloni, L., Pinto, A.F., Moresco, J., Yates III, J.R., da Silva Vaz Jr, Itabajara, Mulenga, A., 2016. Ixodes scapularis tick saliva proteins sequentially secreted every 24 h during blood feeding. PLoS neglected tropical diseases 10, e0004323.

Kim, T.K., Tirloni, L., Radulovic, Z., Lewis, L., Bakshi, M., Hill, C., da Silva Vaz, I., Logullo, C., Termignoni, C., Mulenga, A., 2015b. Conserved Amblyomma americanum

tick Serpin19, an inhibitor of blood clotting factors Xa and XIa, trypsin and plasmin, has anti-haemostatic functions. Int. J. Parasitol. 45, 613-627.

Kim, E., Tyagi, R., Lee, J.Y., Park, J., Kim, Y.R., Beon, J., Chen, P.Y., Cha, J.Y., Snyder, S.H., Kim, S., 2013. Inositol polyphosphate multikinase is a coactivator for serum response factor-dependent induction of immediate early genes. Proc. Natl. Acad. Sci. U. S. A. 110, 19938-19943.

Kim, T.K., Curran, J., Mulenga, A., 2014. Dual silencing of long and short Amblyomma americanum acidic chitinase forms weakens the tick cement cone stability. J. Exp. Biol. 217, 3493-3503.

Kirkland, B.H., Cho, E., Keyhani, N.O., 2004a. Differential susceptibility of Amblyomma maculatum and Amblyomma americanum (Acari: Ixodidea) to the entomopathogenic fungi Beauveria bassiana and Metarhizium anisopliae. Biological Control 31, 414-421.

Kirkland, B.H., Westwood, G.S., Keyhani, N.O., 2004b. Pathogenicity of entomopathogenic fungi Beauveria bassiana and Metarhizium anisopliae to Ixodidae tick species Dermacentor variabilis, Rhipicephalus sanguineus, and Ixodes scapularis. J. Med. Entomol. 41, 705-711.

Kitaura, M., Nakajima, T., Imai, T., Harada, S., Combadiere, C., Tiffany, H.L., Murphy, P.M., Yoshie, O., 1996. Molecular cloning of human eotaxin, an eosinophil-selective CC

chemokine, and identification of a specific eosinophil eotaxin receptor, CC chemokine receptor 3. J. Biol. Chem. 271, 7725-7730.

Kjemtrup, A.M., Conrad, P.A., 2006. A review of the small canine piroplasms from California: Babesia conradae in the literature. Vet. Parasitol. 138, 112-117.

Klompen, J., Black, W., Keirans, J., Oliver Jr, J., 1996. Evolution of ticks. Annu. Rev. Entomol. 41, 141-161.

Konik, P., Slavikova, V., Salát, J., Řezníčková, J., Dvorožňáková, E., Kopecký, J., 2006. Anti- tumour necrosis factor- α activity in Ixodes ricinus saliva. Parasite Immunol. 28, 649-656.

Konnai, S., Nishikado, H., Yamada, S., Imamura, S., Ito, T., Onuma, M., Murata, S., Ohashi, K., 2011. Molecular identification and expression analysis of lipocalins from blood feeding taiga tick, Ixodes persulcatus Schulze. Exp. Parasitol. 127, 467-474.

Kotsyfakis, M., Sa-Nunes, A., Francischetti, I.M., Mather, T.N., Andersen, J.F., Ribeiro, J.M., 2006. Antiinflammatory and immunosuppressive activity of sialostatin L, a salivary cystatin from the tick Ixodes scapularis. J. Biol. Chem. 281, 26298-26307.

Kotsyfakis, M., Schwarz, A., Erhart, J., Ribeiro, J.M., 2015. Tissue- and time-dependent transcription in Ixodes ricinus salivary glands and midguts when blood feeding on the vertebrate host. Sci. Rep. 5, 9103.

Kovář, L., Kopecký, J., Říhová, B., 2001. Salivary gland extract from Ixodes ricinus tick polarizes the cytokine profile toward Th2 and suppresses proliferation of T lymphocytes in human PBMC culture. J. Parasitol. 87, 1342-1348.

Kramer, C.D., Poole, N.M., Coons, L.B., Cole, J.A., 2011. Tick saliva regulates migration, phagocytosis, and gene expression in the macrophage-like cell line, IC-21. Exp. Parasitol. 127, 665-671.

Krause, P.J., Grant-Kels, J.M., Tahan, S.R., Dardick, K.R., Alarcon-Chaidez, F., Bouchard, K., Visini, C., Deriso, C., Foppa, I.M., Wikel, S., 2009. Dermatologic changes induced by repeated Ixodes scapularis bites and implications for prevention of tick-borne infection. Vector-Borne and Zoonotic Diseases 9, 603-610.

Kuchroo, V.K., Das, M.P., Brown, J.A., Ranger, A.M., Zamvil, S.S., Sobel, R.A., Weiner, H.L., Nabavi, N., Glimcher, L.H., 1995. B7-1 and B7-2 costimulatory molecules activate differentially the Th1/Th2 developmental pathways: application to autoimmune disease therapy. Cell 80, 707-718.

Kumar, A., Garg, R., Yadav, C., Vatsya, S., Kumar, R., Sugumar, P., Chandran, D., Mangamoorib, L.N., Bedarkar, S., 2009. Immune responses against recombinant tick antigen, Bm95, for the control of Rhipicephalus (Boophilus) microplus ticks in cattle. Vet. Parasitol. 165, 119-124.

Kumar, V., Sharma, A., 2010. Neutrophils: Cinderella of innate immune system. Int. Immunopharmacol. 10, 1325-1334.

Kýčková, K., Kopecký, J., 2006. Effect of tick saliva on mechanisms of innate immune response against Borrelia afzelii. J. Med. Entomol. 43, 1208-1214.

Labuda, M., Jones, L., Williams, T., Nuttall, P., 1993. Enhancement of tick- borne encephalitis virus transmission by tick salivary gland extracts. Med. Vet. Entomol. 7, 193-196.

Labuda, M., Nuttall, P., 2004. Tick-borne viruses. Parasitology 129, S221-S245.

Langhansova, H., Bopp, T., Schmitt, E., Kopecký, J., 2015. Tick saliva increases production of three chemokines including monocyte chemoattractant protein- 1, a histamine- releasing cytokine. Parasite Immunol. 37, 92-96.

Lathrop, S.L., Ball, R., Haber, P., Mootrey, G.T., Braun, M.M., Shadomy, S.V., Ellenberg, S.S., Chen, R.T., Hayes, E.B., 2002. Adverse event reports following vaccination for Lyme disease: December 1998–July 2000. Vaccine 20, 1603-1608.

Leboulle, G., Crippa, M., Decrem, Y., Mejri, N., Brossard, M., Bollen, A., Godfroid, E., 2002. Characterization of a novel salivary immunosuppressive protein from Ixodes ricinus ticks. J. Biol. Chem. 277, 10083-10089.

Lepidi, H., Bunnell, J.E., Martin, M.E., Madigan, J.E., Stuen, S., Dumler, J.S., 2000. Comparative pathology, and immunohistology associated with clinical illness after Ehrlichia phagocytophila-group infections. Am. J. Trop. Med. Hyg. 62, 29-37.

Lewis, L.A., Radulović, Ž.M., Kim, T.K., Porter, L.M., Mulenga, A., 2015. Identification of 24h Ixodes scapularis immunogenic tick saliva proteins. Ticks and tick-borne diseases 6, 424-434.

Liu, Y., Li, Q., Hu, W., Wu, J., Wang, Y., Mei, L., Walker, D.H., Ren, J., Wang, Y., Yu, X., 2012. Person-to-person transmission of severe fever with thrombocytopenia syndrome virus. Vector-Borne and Zoonotic Diseases 12, 156-160.

Li, J., Du, Y., Yan, Z., Yan, J., Zhuansun, Y., Chen, R., Zhang, W., Feng, S., Ran, P., 2016. CD80 and CD86 knockdown in dendritic cells regulates Th1/Th2 cytokine production in asthmatic mice. Experimental and therapeutic medicine 11, 878-884.

Lieskovska, J., Kopecký, J., 2012. Tick saliva suppresses IFN signalling in dendritic cells upon Borrelia afzelii infection. Parasite Immunol. 34, 32-39.

Lieskovska, J., Kopecky, J., 2012. Effect of tick saliva on signalling pathways activated by TLR- 2 ligand and Borrelia afzelii in dendritic cells. Parasite Immunol. 34, 421-429.

Lieskovska, J., Palenikova, J., Širmarová, J., Elsterova, J., Kotsyfakis, M., Campos Chagas, A., Calvo, E., Růžek, D., Kopecký, J., 2015. Tick salivary cystatin sialostatin L2 suppresses IFN responses in mouse dendritic cells. Parasite Immunol. 37, 70-78.

Lieskovská, J., Páleníková, J., Langhansová, H., Chagas, A.C., Calvo, E., Kotsyfakis, M., Kopecký, J., 2015. Tick sialostatins L and L2 differentially influence dendritic cell responses to Borrelia spirochetes. Parasites & vectors 8, 275.

Lima e Silva, M., Szabo, M., Bechara, G., 2004. Microscopic Features of Tick- Bite Lesions in Anteaters and Armadillos: Emas National Park and the Pantanal Region of Brazil. Ann. N. Y. Acad. Sci. 1026, 235-241.

Littman, M.P., Goldstein, R.E., Labato, M.A., Lappin, M.R., Moore, G.E., 2006. ACVIM small animal consensus statement on Lyme disease in dogs: diagnosis, treatment, and prevention. Journal of veterinary internal medicine 20, 422-434.

Liu, J., Renneker, S., Beyer, D., Kullmann, B., Seitzer, U., Ahmed, J., Bakheit, M., 2014. Identification and partial characterization of a Salp15 homolog from Ixodes ricinus. Ticks and tick-borne diseases 5, 318-322.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. Methods 25, 402-408.

Longo, P.A., Kavran, J.M., Kim, M.S., Leahy, D.J., 2013. Transient mammalian cell transfection with polyethylenimine (PEI). Methods Enzymol. 529, 227-240.

Lu, Y., Yeh, W., Ohashi, P.S., 2008. LPS/TLR4 signal transduction pathway. Cytokine 42, 145-151.

Mahoney, D.F., Mirre, G.B., 1977. The selection of larvae of Boophilus microplus infected with Babesia bovis (syn B argentina). Res. Vet. Sci. 23, 126-127.

Mans, B.J., De Klerk, D., Pienaar, R., Latif, A.A., 2011. Nuttalliella namaqua: a living fossil and closest relative to the ancestral tick lineage: implications for the evolution of blood-feeding in ticks. PloS one 6, e23675.

Marcelino, I., de Almeida, A.M., Ventosa, M., Pruneau, L., Meyer, D.F., Martinez, D., Lefrançois, T., Vachiéry, N., Coelho, A.V., 2012. Tick-borne diseases in cattle: Applications of proteomics to develop new generation vaccines. Journal of proteomics 75, 4232-4250.

Marchal, C., Schramm, F., Kern, A., Luft, B.J., Yang, X., Schuijt, T.J., Hovius, J.W., Jaulhac, B., Boulanger, N., 2011. Antialarmin effect of tick saliva during the transmission of Lyme disease. Infect. Immun. 79, 774-785.

Martinez, F.O., Gordon, S., 2014. The M1 and M2 paradigm of macrophage activation: time for reassessment. F1000 Prime Rep. 6, 13-13. eCollection 2014.

Matsuno, K., Orba, Y., Maede-White, K., Scott, D., Feldmann, F., Liang, M., Ebihara, H., 2017. Animal models of emerging tick-borne phleboviruses: determining target cells in a lethal model of SFTSV infection. Frontiers in microbiology 8.

Maxwell, S., Stoklasek, T., Dash, Y., Macaluso, K., Wikel, S., 2005. Tick modulation of the in-vitro expression of adhesion molecules by skin-derived endothelial cells. Annals of Tropical Medicine & Parasitology 99, 661-672.

Mazur, P.K., Herner, A., Mello, S.S., Wirth, M., Hausmann, S., Sanchez-Rivera, F.J., Lofgren, S.M., Kuschma, T., Hahn, S.A., Vangala, D., Trajkovic-Arsic, M., Gupta, A., Heid, I., Noel, P.B., Braren, R., Erkan, M., Kleeff, J., Sipos, B., Sayles, L.C., Heikenwalder, M., Hessmann, E., Ellenrieder, V., Esposito, I., Jacks, T., Bradner, J.E., Khatri, P., Sweet-Cordero, E.A., Attardi, L.D., Schmid, R.M., Schneider, G., Sage, J., Siveke, J.T., 2015. Combined inhibition of BET family proteins and histone deacetylases as a potential epigenetics-based therapy for pancreatic ductal adenocarcinoma. Nat. Med. 21, 1163-1171.

Mbow, M., Christe, M., Rutti, B., Brossard, M., 1994. Absence of acquired resistance to nymphal Ixodes ricinus ticks in BALB/c mice developing cutaneous reactions. J. Parasitol. , 81-87.

Meddeb, M., Carpentier, W., Cagnard, N., Nadaud, S., Grillon, A., Barthel, C., De Martino, S.J., Jaulhac, B., Boulanger, N., Schramm, F., 2016. Homogeneous Inflammatory Gene Profiles Induced in Human Dermal Fibroblasts in Response to the Three Main Species of Borrelia burgdorferi sensu lato. PloS one 11, e0164117.

Medzhitov, R., Janeway, C.A., 1997. Innate immunity: impact on the adaptive immune response. Curr. Opin. Immunol. 9, 4-9. Medzhitov, R., 2001. Toll-like receptors and innate immunity. Nature reviews.Immunology 1, 135.

Mejri, N., Brossard, M., 2007. Splenic dendritic cells pulsed with Ixodes ricinus tick saliva prime naive CD4 T to induce Th2 cell differentiation in vitro and in vivo. Int. Immunol. 19, 535-543.

Merino, O., Almazán, C., Canales, M., Villar, M., Moreno-Cid, J.A., Estrada-Peña, A., Kocan, K.M., de la Fuente, J., 2011. Control of Rhipicephalus (Boophilus) microplus infestations by the combination of subolesin vaccination and tick autocidal control after subolesin gene knockdown in ticks fed on cattle. Vaccine 29, 2248-2254.

Miura, K., Matsuo, J., Rahman, M.A., Kumagai, Y., Li, X., Rikihisa, Y., 2011. Ehrlichia chaffeensis induces monocyte inflammatory responses through MyD88, ERK, and NF-kappaB but not through TRIF, interleukin-1 receptor 1 (IL-1R1)/IL-18R1, or toll-like receptors. Infect. Immun. 79, 4947-4956.

Montenagro- James, S., Toro, M., Leon, E., Guillen, A., Lopez, R., Lopez, W., 1992. Immunization of cattle with an inactivated polyvalent vaccine against anaplasmosis and babesiosis. Ann. N. Y. Acad. Sci. 653, 112-121.

Moreira, H.N.S., Barcelos, R.M., Vidigal, P.M.P., Klein, R.C., Montandon, C.E., Maciel, T.E.F., Carrizo, J.F.A., de Lima, Paulo Henrique Costa, Soares, A.C., Martins, M.M., 2017. A deep insight into the whole transcriptome of midguts, ovaries and salivary glands of the Amblyomma sculptum tick. Parasitol. Int. 66, 64-73.

Morgado, P., Sudarshana, D.M., Gov, L., Harker, K.S., Lam, T., Casali, P., Boyle, J.P., Lodoen, M.B., 2014. Type II Toxoplasma gondii induction of CD40 on infected macrophages enhances interleukin-12 responses. Infect. Immun. 82, 4047-4055.

Morris, C.J., 2003. Carrageenan-induced paw edema in the rat and mouse. Inflammation protocols , 115-121.

Mudenda, L., Pierlé, S.A., Turse, J.E., Scoles, G.A., Purvine, S.O., Nicora, C.D., Clauss, T.R., Ueti, M.W., Brown, W.C., Brayton, K.A., 2014. Proteomics informed by transcriptomics identifies novel secreted proteins in Dermacentor andersoni saliva. Int. J. Parasitol. 44, 1029-1037.

Mulenga, A., Kim, T., Ibelli, A., 2013. Amblyomma americanum tick saliva serine protease inhibitor 6 is a cross- class inhibitor of serine proteases and papain- like cysteine proteases that delays plasma clotting and inhibits platelet aggregation. Insect Mol. Biol. 22, 306-319.

Mulenga, A., Blandon, M., Khumthong, R., 2007. The molecular basis of the Amblyomma americanum tick attachment phase. Experimental and Applied Acarology 41, 267-287.

Mulenga, A., Sugimoto, C., Ohashi, K., Onuma, M., 2000. Characterization of an 84 kDa protein inducing an immediate hypersensitivity reaction in rabbits sensitized to Haemaphysalis longicornis ticks. Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease 1501, 219-226.

Mulenga, A., Sugino, M., Nakajima, M., Sugimoto, C., Onuma, M., 2001. Tick-Encoded serine proteinase inhibitors (serpins); potential target antigens for tick vaccine development. Journal of Veterinary Medical Science 63, 1063-1069.

Mulenga, A., Khumthong, R., 2010. Silencing of three Amblyomma americanum (L.) insulin-like growth factor binding protein-related proteins prevents ticks from feeding to repletion. J. Exp. Biol. 213, 1153-1161.

Mulenga, A., Khumthong, R., Blandon, M.A., 2007. Molecular and expression analysis of a family of the Amblyomma americanum tick Lospins. J. Exp. Biol. 210, 3188-3198.

Mulenga, A., Sugimoto, C., Sako, Y., Ohashi, K., Musoke, A., Shubash, M., Onuma, M., 1999. Molecular characterization of a Haemaphysalis longicornis tick salivary gland-associated 29-kilodalton protein and its effect as a vaccine against tick infestation in rabbits. Infect. Immun. 67, 1652-1658.

Moon, R., Mullen, G., Durden, L., 2002. Medical and veterinary entomology. Med. Vet. Entomol. .

Murooka, T.T., Rahbar, R., Platanias, L.C., Fish, E.N., 2008. CCL5-mediated T-cell chemotaxis involves the initiation of mRNA translation through mTOR/4E-BP1. Blood 111, 4892-4901.

Naredi, P.L., Lindnér, P.G., Holmberg, S.B., Stenram, U., Peterson, A., Hafström, L.R., 1993. The effects of tumour necrosis factor alpha on the vascular bed and blood flow in an experimental rat hepatoma. International journal of cancer 54, 645-649.

Nathavitharana, R.R., Mitty, J.A., 2015. Diseases from North America: focus on tick-borne infections. Clin. Med. (Lond) 15, 74-77.

Njongmeta, L.M., Bray, J., Davies, C.J., Davis, W.C., Howard, C.J., Hope, J.C., Palmer, G.H., Brown, W.C., Mwangi, W., 2012. CD205 antigen targeting combined with dendritic cell recruitment factors and antigen-linked CD40L activation primes and expands significant antigen-specific antibody and CD4 T cell responses following DNA vaccination of outbred animals. Vaccine 30, 1624-1635.

Nuttall, P., Labuda, M., 2008. 10• Saliva-assisted transmission of tick-borne pathogens. Biology, Disease and Control , 205.

Ogden, N.H., Casey, A.N., Woldehiwet, Z., French, N.P., 2003. Transmission of Anaplasma phagocytophilum to Ixodes ricinus ticks from sheep in the acute and post-acute phases of infection. Infect. Immun. 71, 2071-2078.

Oliveira, C.J.F., Carvalho, W.A., Garcia, G.R., Gutierrez, F.R., de Miranda Santos, Isabel KF, Silva, J.S., Ferreira, B.R., 2010. Tick saliva induces regulatory dendritic cells: MAP-kinases and Toll-like receptor-2 expression as potential targets. Vet. Parasitol. 167, 288-297.

Oliveira, C.J.F., Cavassani, K.A., Moré, D.D., Garlet, G.P., Aliberti, J.C., Silva, J.S., Ferreira, B.R., 2008. Tick saliva inhibits the chemotactic function of MIP-1α and selectively impairs chemotaxis of immature dendritic cells by down-regulating cell-surface CCR5. Int. J. Parasitol. 38, 705-716.

Oliveira, C., Anatriello, E., de Miranda-Santos, I., Francischetti, I., Sá-Nunes, A., Ferreira, B., Ribeiro, J., 2013. Proteome of Rhipicephalus sanguineus tick saliva induced by the secretagogues pilocarpine and dopamine. Ticks and tick-borne diseases 4, 469-477.

Oliveira, C.J., Sa-Nunes, A., Francischetti, I.M., Carregaro, V., Anatriello, E., Silva, J.S., Santos, I.K., Ribeiro, J.M., Ferreira, B.R., 2011. Deconstructing tick saliva: non-protein molecules with potent immunomodulatory properties. J. Biol. Chem. 286, 10960-10969.

Ōyanagui, Y., Sato, S., Inoue, M., 1991. Inhibition of carrageenan-induced paw edema by superoxide dismutase that binds to heparan sulfates on vascular endothelial cells. Biochem. Pharmacol. 42, 991-995.

Öz, B.E., Çitoğlu, G.S., Akkol, E.K., Süntar, İ., Acıkara, Ö.B., 2017. Isoflavonoids as wound healing agents from Ononidis Radix. J. Ethnopharmacol.

Parameswaran, N., Patial, S., 2010. Tumor necrosis factor-α signaling in macrophages. Critical Reviews™ in Eukaryotic Gene Expression 20.

Parihar, A., Eubank, T.D., Doseff, A.I., 2010. Monocytes and macrophages regulate immunity through dynamic networks of survival and cell death. J. Innate Immun. 2, 204-215.

Parola, P., Raoult, D., 2001. Ticks and tickborne bacterial diseases in humans: an emerging infectious threat. Clinical infectious diseases 32, 897-928.

Pavela, R., Canale, A., Mehlhorn, H., Benelli, G., 2016. Application of ethnobotanical repellents and acaricides in prevention, control and management of livestock ticks: a review. Res. Vet. Sci. 109, 1-9.

Pechová, J., Kopecký, J., Salát, J., 2004. Effect of tick salivary gland extract on the cytokine production by mouse epidermal cells. Folia Parasitol. 51, 367.

Poole, N.M., Mamidanna, G., Smith, R.A., Coons, L.B., Cole, J.A., 2013. Prostaglandin E 2 in tick saliva regulates macrophage cell migration and cytokine profile. Parasites & vectors 6, 261.

Porter, L., Radulović, Ž., Kim, T., Braz, G.R., Vaz, I.D.S., Mulenga, A., 2015. Bioinformatic analyses of male and female Amblyomma americanum tick expressed serine protease inhibitors (serpins). Ticks and tick-borne diseases 6, 16-30.

Porter, L.M., Radulović, Ž.M., Mulenga, A., 2017. A repertoire of protease inhibitor families in Amblyomma americanum and other tick species: inter-species comparative analyses. Parasites & vectors 10, 152.

Portolano, N., Watson, P.J., Fairall, L., Millard, C.J., Milano, C.P., Song, Y., Cowley, S.M., Schwabe, J.W., 2014. Recombinant protein expression for structural biology in HEK 293F suspension cells: a novel and accessible approach. J. Vis. Exp. (92):e51897. doi, e51897.

Preston, S.G., Majtán, J., Kouremenou, C., Rysnik, O., Burger, L.F., Cruz, A.C., Guzman, M.C., Nunn, M.A., Paesen, G.C., Nuttall, P.A., 2013. Novel immunomodulators from hard ticks selectively reprogramme human dendritic cell responses. PLoS pathogens 9, e1003450.

Prevot, P., Couvreur, B., Denis, V., Brossard, M., Vanhamme, L., Godfroid, E., 2007. Protective immunity against Ixodes ricinus induced by a salivary serpin. Vaccine 25, 3284-3292.

Radley, D., Brown, C., Burridge, M., Cunningham, M., Kirimi, I., Purnell, R., Young, A., 1975. East Coast fever: 1. Chemoprophylactic immunization of cattle against Theileria parva (Muguga) and five theilerial strains. Vet. Parasitol. 1, 35-41.

Radulović, Ž.M., Mulenga, A., 2017. Heparan sulfate/heparin glycosaminoglycan binding alters inhibitory profile and enhances anticoagulant function of conserved Amblyomma americanum tick saliva serpin 19. Insect Biochem. Mol. Biol. 80, 1-10.

Radulović, Ž.M., Porter, L.M., Kim, T.K., Bakshi, M., Mulenga, A., 2015. Amblyomma americanum tick saliva insulin- like growth factor binding protein- related protein 1 binds insulin but not insulin- like growth factors. Insect Mol. Biol. 24, 539-550.

Rechav, Y., Dauth, J., 1987. Development of resistance in rabbits to immature stages of the Ixodid tick Rhipicephalus appendiculatus. Med. Vet. Entomol. 1, 177-183.

Reichard, M.V., Meinkoth, J.H., Edwards, A.C., Snider, T.A., Kocan, K.M., Blouin, E.F., Little, S.E., 2009. Transmission of Cytauxzoon felis to a domestic cat by Amblyomma americanum. Vet. Parasitol. 161, 110-115.

Ribeiro, J.M., 1989. Role of saliva in tick/host interactions. Experimental and Applied Acarology 7, 15-20.

Ribeiro, J.M., Francischetti, I.M., 2003. Role of Arthropod Saliva in Blood Feeding: Sialome and Post-Sialome Perspectives*. Annu. Rev. Entomol. 48, 73-88.

Ribeiro, J.M., Slovák, M., Francischetti, I.M., 2017. An insight into the sialome of Hyalomma excavatum. Ticks and tick-borne diseases 8, 201-207.

Ribeiro, J.C., Evans, P.M., MacSwain, J., Sauer, J., 1992. Amblyomma americanum: characterization of salivary prostaglandins E2 and F2α by RP-HPLC/bioassay and gas chromatography-mass spectrometry. Exp. Parasitol. 74, 112-116.

Ribeiro, J.M., Makoul, G.T., Levine, J., Robinson, D.R., Spielman, A., 1985. Antihemostatic, antiinflammatory, and immunosuppressive properties of the saliva of a tick, Ixodes dammini. J. Exp. Med. 161, 332-344.

Richards, G.A., 2015. Nososcomial transmission of viral haemorrhagic fever in South Africa. SAMJ: South African Medical Journal 105, 709-712.

Richards, S.L., Langley, R., Apperson, C.S., Watson, E., 2017. Do Tick Attachment Times Vary between Different Tick-Pathogen Systems? Environments 4, 37.

Rodriguez, M., Penichet, M., Mouris, A., Labarta, V., Luaces, L.L., Rubiera, R., Cordoves, C., Sanchez, P., Ramos, E., Soto, A., 1995. Control of Boophilus microplus populations in grazing cattle vaccinated with a recombinant Bm86 antigen preparation. Vet. Parasitol. 57, 339-349.

Rodriguez, M., Massard, C.L., da Fonseca, A.H., Ramos, N.F., Machado, H., Labarta, V., de la Fuente, J., 1995. Effect of vaccination with a recombinant Bm86 antigen preparation on natural infestations of Boophilus microplus in grazing dairy and beef pure and cross-bred cattle in Brazil. Vaccine 13, 1804-1808.

Rosario-Cruz, R., Almazan, C., Miller, R.J., Dominguez-Garcia, D.I., Hernandez-Ortiz, R., de la Fuente, J., 2009. Genetic basis and impact of tick acaricide resistance. Front. Biosci. (Landmark Ed) 14, 2657-2665.

Rose, C.D., Fawcett, P.T., Gibney, K.M., 2001. Arthritis following recombinant outer surface protein A vaccination for Lyme disease. J. Rheumatol. 28, 2555-2557.

Sadeghi, M., Eckerle, I., Daniel, V., Burkhardt, U., Opelz, G., Schnitzler, P., 2011. Cytokine expression during early and late phase of acute Puumala hantavirus infection. BMC immunology 12, 65.

Sahni, S.K., Rydkina, E., 2009. Host-cell interactions with pathogenic Rickettsia species.

Saimo, M., Odongo, D., Mwaura, S., Vlak, J., Musoke, A., Lubega, G., Bishop, R., van Oers, M., 2011. Recombinant Rhipicephalus appendiculatus gut (Ra86) and salivary gland cement (Trp64) proteins as candidate antigens for inclusion in tick vaccines: protective effetcs of Ra86 on infestation with adult R. appendiculatus. Vaccine: Development and Therapy 1, 15-23.

Sakat, S.S., Mani, K., Demidchenko, Y.O., Gorbunov, E.A., Tarasov, S.A., Mathur, A., Epstein, O.I., 2014. Release-active dilutions of diclofenac enhance anti-inflammatory effect of diclofenac in carrageenan-induced rat paw edema model. Inflammation 37, 1-9.

Salomon, B., Bluestone, J.A., 2001. Complexities of CD28/B7: CTLA-4 costimulatory pathways in autoimmunity and transplantation. Annu. Rev. Immunol. 19, 225-252.

Santin, A.D., Hermonat, P.L., Ravaggi, M.C., Chiriva-Internat, M., Cannon, M.J., Hiserodt, J.C., Pecorell, S., Parham, G.P., 1999. Expression of surface antigens during the differentiation of human dendritic cells vs macrophages from blood monocytes in vitro. Immunobiology 200, 187-204.

Sa-Nunes, A., Bafica, A., Lucas, D.A., Conrads, T.P., Veenstra, T.D., Andersen, J.F., Mather, T.N., Ribeiro, J.M., Francischetti, I.M., 2007. Prostaglandin E2 is a major inhibitor of dendritic cell maturation and function in Ixodes scapularis saliva. J. Immunol. 179, 1497-1505.

Saraiva, D.G., Soares, H.S., Soares, J.F., Labruna, M.B., 2014. Feeding period required by Amblyomma aureolatum ticks for transmission of Rickettsia rickettsii to vertebrate hosts. Emerg. Infect. Dis. 20, 1504-1510.

Scholl, D.C., Embers, M.E., Caskey, J.R., Kaushal, D., Mather, T.N., Buck, W.R., Morici, L.A., Philipp, M.T., 2016. Immunomodulatory effects of tick saliva on dermal cells exposed to Borrelia burgdorferi, the agent of Lyme disease. Parasites & vectors 9, 394.

Schwartz, B.S., Ribeiro, J.M., Goldstein, M.D., 1990. Anti-tick antibodies: an epidemiologic tool in Lyme disease research. Am. J. Epidemiol. 132, 58-66.

Schwarz, A., von Reumont, B.M., Erhart, J., Chagas, A.C., Ribeiro, J.M., Kotsyfakis, M., 2013. De novo Ixodes ricinus salivary gland transcriptome analysis using two next-generation sequencing methodologies. FASEB J. 27, 4745-4756.

Schwarz, A., Tenzer, S., Hackenberg, M., Erhart, J., Gerhold-Ay, A., Mazur, J., Kuharev, J., Ribeiro, J.M., Kotsyfakis, M., 2014. A systems level analysis reveals transcriptomic and proteomic complexity in Ixodes ricinus midgut and salivary glands during early attachment and feeding. Mol. Cell. Proteomics 13, 2725-2735.

Shapiro, S.Z., Voigt, W.P., Fujisaki, K., 1986. Tick antigens recognized by serum from a guinea pig resistant to infestation with the tick Rhipicephalus appendiculatus. J. Parasitol. , 454-463.

Sica, A., Mantovani, A., 2012. Macrophage plasticity and polarization: in vivo veritas. J. Clin. Invest. 122, 787-795.

Šimo, L., Kazimirova, M., Richardson, J., Bonnet, S.I., 2017. The essential role of tick salivary glands and saliva in tick feeding and pathogen transmission. Frontiers in Cellular and Infection Microbiology 7.

Skallova, A., Iezzi, G., Ampenberger, F., Kopf, M., Kopecky, J., 2008. Tick saliva inhibits dendritic cell migration, maturation, and function while promoting development of Th2 responses. J. Immunol. 180, 6186-6192.

Slámová, M., Skallová, A., Palenikova, J., Kopecký, J., 2011. Effect of tick saliva on immune interactions between Borrelia afzelii and murine dendritic cells. Parasite Immunol. 33, 654-660.

Slawek, A., Maj, T., Chelmonska- Soyta, A., 2013. CD40, CD80, and CD86 costimulatory molecules are differentially expressed on murine splenic antigen-presenting cells during the pre- implantation period of pregnancy, and they modulate regulatory T- cell abundance, peripheral cytokine response, and pregnancy outcome. American Journal of Reproductive Immunology 70, 116-126.

Somoza, C., Lanier, L., 1995. T-cell costimulation via CD28-CD80/CD86 and CD40-CD40 ligand interactions. Res. Immunol. 146, 171-176.

Sonenshine, D.E., 1991. Biology of Ticks: Vol. 1. Oxford University Press New York.

Starkey, L., Barrett, A., Beall, M., Chandrashekar, R., Thatcher, B., Tyrrell, P., Little, S., 2015. Persistent Ehrlichia ewingii infection in dogs after natural tick infestation. Journal of veterinary internal medicine 29, 552-555.

Steere, A.C., Sikand, V.K., Meurice, F., Parenti, D.L., Fikrig, E., Schoen, R.T., Nowakowski, J., Schmid, C.H., Laukamp, S., Buscarino, C., 1998. Vaccination against Lyme disease with recombinant Borrelia burgdorferi outer-surface lipoprotein A with adjuvant. N. Engl. J. Med. 339, 209-215.

Sternberg, E.M., 2006. Neural regulation of innate immunity: a coordinated nonspecific host response to pathogens. Nature Reviews Immunology 6, 318-328.

Sugino, M., Imamura, S., Mulenga, A., Nakajima, M., Tsuda, A., Ohashi, K., Onuma, M., 2003. A serine proteinase inhibitor (serpin) from ixodid tick Haemaphysalis longicornis; cloning and preliminary assessment of its suitability as a candidate for a tick vaccine. Vaccine 21, 2844-2851.

Szabó, M., Morelli, J., Bechara, G., 1995. Cutaneous hypersensitivity induced in dogs and guinea-pigs by extracts of the tick Rhipicephalus sanguineus (Acari: Ixodidae). Exp. Appl. Acarol. 19, 723-730.

Tirloni, L., Islam, M.S., Kim, T.K., Diedrich, J.K., Yates, J.R., Pinto, A.F., Mulenga, A., You, M., Vaz, I.D.S., 2015. Saliva from nymph and adult females of Haemaphysalis longicornis: a proteomic study. Parasites & vectors 8, 1.

Tirloni, L., Kim, T.K., Coutinho, M.L., Ali, A., Seixas, A., Termignoni, C., Mulenga, A., da Silva Vaz, I., 2016. The putative role of Rhipicephalus microplus salivary serpins in the tick-host relationship. Insect Biochem. Mol. Biol. 71, 12-28.

Tirloni, L., Reck, J., Terra, R.M.S., Martins, J.R., Mulenga, A., Sherman, N.E., Fox, J.W., Yates III, J.R., Termignoni, C., Pinto, A.F., 2014. Proteomic analysis of cattle tick Rhipicephalus (Boophilus) microplus saliva: a comparison between partially and fully engorged females. PloS one 9, e94831.

Townsend, R.L., Moritz, E.D., Fialkow, L.B., Berardi, V., Stramer, S.L., 2014. Probable transfusion-transmission of Anaplasma phagocytophilum by leukoreduced platelets. Transfusion 54, 2828-2832.

Trager, W., 1939. Acquired immunity to ticks. J. Parasitol. 25, 57-81.

Uilenberg, G., Dobbelaere, D., De Gee, A., Koch, H., 1993. Progress in research on tick-borne diseases: Theileriosis and heartwater. Vet. Q. 15, 48-54.

Vachiery, N., Puech, C., Cavelier, P., Rodrigues, V., Aprelon, R., Lefrançois, T., Martinez, D., Epardaud, M., 2015. An in vitro model to assess the immunosuppressive effect of tick saliva on the mobilization of inflammatory monocyte-derived cells. Vet. Res. 46, 117.

Vachiery, N., Puech, C., Cavelier, P., Rodrigues, V., Aprelon, R., Lefrançois, T., Martinez, D., Epardaud, M., 2015. An in vitro model to assess the immunosuppressive

effect of tick saliva on the mobilization of inflammatory monocyte-derived cells. Vet. Res. 46, 117.

Vajja, B.N., Juluri, S., Kumari, M., Kole, L., Chakrabarti, R., Joshi, V.D., 2004. Lipopolysaccharide-induced paw edema model for detection of cytokine modulating anti-inflammatory agents. Int. Immunopharmacol. 4, 901-909.

Valle, M.R., Mèndez, L., Valdez, M., Redondo, M., Espinosa, C.M., Vargas, M., Cruz, R.L., Barrios, H.P., Seoane, G., Ramirez, E.S., 2004. Integrated control of Boophilus microplus ticks in Cuba based on vaccination with the anti-tick vaccine Gavac TM. Experimental and Applied Acarology 34, 375-382.

Vančová, I., Hajnická, V., Slovák, M., Kocáková, P., Paesen, G., Nuttall, P., 2010. Evasin- 3- like anti- chemokine activity in salivary gland extracts of ixodid ticks during blood- feeding: a new target for tick control. Parasite Immunol. 32, 460-463.

Vančová, I., Slovak, M., Hajnicka, V., Labuda, M., Šimo, L., Peterkova, K., Hails, R., Nuttall, P., 2007. Differential anti- chemokine activity of Amblyomma variegatum adult ticks during blood- feeding. Parasite Immunol. 29, 169-177.

Varela-Stokes, A., 2007. Transmission of Ehrlichia chaffeensis from lone star ticks (Amblyomma americanum) to white-tailed deer (Odocoileus virginianus). J. Wildl. Dis. 43, 376-381.

Vercruysse, J., Knox, D.P., Schetters, T.P., Willadsen, P., 2004. Veterinary parasitic vaccines: pitfalls and future directions. Trends Parasitol. 20, 488-492.

Vesely, D.L., Fish, D., Shlomchik, M.J., Kaplan, D.H., Bockenstedt, L.K., 2009. Langerhans cell deficiency impairs Ixodes scapularis suppression of Th1 responses in mice. Infect. Immun. 77, 1881-1887.

Vicente- Suarez, I., Takahashi, Y., Cheng, F., Horna, P., Wang, H.W., Wang, H., Sotomayor, E.M., 2007. Identification of a novel negative role of flagellin in regulating IL- 10 production. Eur. J. Immunol. 37, 3164-3175.

Villar, M., Popara, M., Mangold, A.J., de la Fuente, J., 2014. Comparative proteomics for the characterization of the most relevant Amblyomma tick species as vectors of zoonotic pathogens worldwide. Journal of proteomics 105, 204-216.

Vora, A., Taank, V., Dutta, S.M., Anderson, J.F., Fish, D., Sonenshine, D.E., Catravas, J.D., Sultana, H., Neelakanta, G., 2017. Ticks elicit variable fibrinogenolytic activities upon feeding on hosts with different immune backgrounds. Sci. Rep. 7, 44593.

Waisberg, M., Molina-Cruz, A., Mizurini, D.M., Gera, N., Sousa, B.C., Ma, D., Leal, A.C., Gomes, T., Kotsyfakis, M., Ribeiro, J.M., 2014. Plasmodium falciparum infection induces expression of a mosquito salivary protein (Agaphelin) that targets neutrophil function and inhibits thrombosis without impairing hemostasis. PLoS pathogens 10, e1004338.

Wang, X., Shaw, D.K., Sakhon, O.S., Snyder, G.A., Sundberg, E.J., Santambrogio, L., Sutterwala, F.S., Dumler, J.S., Shirey, K.A., Perkins, D.J., Richard, K., Chagas, A.C., Calvo, E., Kopecky, J., Kotsyfakis, M., Pedra, J.H., 2016. The Tick Protein Sialostatin L2 Binds to Annexin A2 and Inhibits NLRC4-Mediated Inflammasome Activation. Infect. Immun. 84, 1796-1805.

Weng, M., Huntley, D., Huang, I.F., Foye-Jackson, O., Wang, L., Sarkissian, A., Zhou, Q., Walker, W.A., Cherayil, B.J., Shi, H.N., 2007. Alternatively activated macrophages in intestinal helminth infection: effects on concurrent bacterial colitis. J. Immunol. 179, 4721-4731.

Wikel, S.K., 1996. Host immunity to ticks. Annu. Rev. Entomol. 41, 1-22.

Wikel, S.K., 1999. Tick modulation of host immunity: an important factor in pathogen transmission. Int. J. Parasitol. 29, 851-859.

Wikel, S., 2013. Ticks and tick-borne pathogens at the cutaneous interface: host defenses, tick countermeasures, and a suitable environment for pathogen establishment. Front. Microbiol. 4, 337.

Willadsen, P., 2004. Anti-tick vaccines. Parasitol. 129 Suppl. S367-S387 .

Willadsen, P., Bird, P., Cobon, G., Hungerford, J., 1995. Commercialisation of a recombinant vaccine against Boophilus microplus. Parasitology 110, S43-S50.

Willadsen, P., Kemp, D., 1988. Vaccination with ‘concealed’antigens for tick control. Parasitology Today 4, 196-198.

Winter, C., Risley, E., Nuss, O., 1962. Carrageenin-induced inflammation in the hind limb of the rat.

Wolpe, S.D., Davatelis, G., Sherry, B., Beutler, B., Hesse, D.G., Nguyen, H.T., Moldawer, L.L., Nathan, C.F., Lowry, S.F., Cerami, A., 1988. Macrophages secrete a novel heparin-binding protein with inflammatory and neutrophil chemokinetic properties. J. Exp. Med. 167, 570-581.

Wu, J., Wang, Y., Liu, H., Yang, H., Ma, D., Li, J., Li, D., Lai, R., Yu, H., 2010. Two immunoregulatory peptides with antioxidant activity from tick salivary glands. J. Biol. Chem. 285, 16606-16613.

Yadav, P.D., Patil, D.Y., Shete, A.M., Kokate, P., Goyal, P., Jadhav, S., Sinha, S., Zawar, D., Sharma, S.K., Kapil, A., 2016. Nosocomial infection of CCHF among health care workers in Rajasthan, India. BMC infectious diseases 16, 624.

Yamamoto, H., Omelchenko, I., Shi, X., Nuttall, A.L., 2009. The influence of NF- κB signal- transduction pathways on the murine inner ear by acoustic overstimulation. J. Neurosci. Res. 87, 1832-1840.

Yamashita, T., Sasaki, N., Kasahara, K., Hirata, K., 2015. Anti-inflammatory and immune-modulatory therapies for preventing atherosclerotic cardiovascular disease. J. Cardiol. 66, 1-8.

Yun, S.M., Lee, W.G., Ryou, J., Yang, S.C., Park, S.W., Roh, J.Y., Lee, Y.J., Park, C., Han, M.G., 2014. Severe fever with thrombocytopenia syndrome virus in ticks collected from humans, South Korea, 2013. Emerg. Infect. Dis. 20, 1358-1361.

Zavašnik-Bergant, T., Vidmar, R., Sekirnik, A., Fonović, M., Salát, J., Grunclová, L., Kopáček, P., Turk, B., 2017. Salivary Tick Cystatin OmC2 Targets Lysosomal Cathepsins S and C in Human Dendritic Cells. Frontiers in cellular and infection microbiology 7.

Zhan, H., Towler, H.M., Calder, V.L., 2003. The immunomodulatory role of human conjunctival epithelial cells. Invest. Ophthalmol. Vis. Sci. 44, 3906-3910.